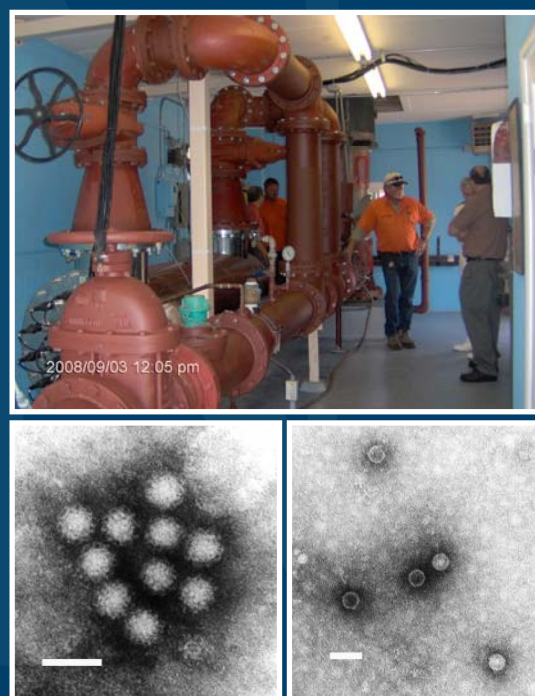


Method 1615

Measurement of Enterovirus and Norovirus Occurrence in Water by Culture and RT-qPCR



METHOD 1615. Enterovirus and Norovirus occurrence in water

Cover:

Top picture: Prairie Du Sac, WI Pump house, courtesy of Dr. Mark Borchardt

Bottom left picture: norovirus, courtesy of Fred P. Williams; Bar = 50 nanometers

Bottom right picture: poliovirus, courtesy of Fred P. Williams; Bar = 50 nanometers

METHOD 1615. Enterovirus and Norovirus occurrence in water

METHOD 1615

MEASUREMENT OF ENTEROVIRUS AND NOROVIRUS OCCURRENCE IN WATER BY CULTURE AND RT-qPCR

Version 1.0

December 2010

AUTHORS

G. S. Fout,¹ N. E. Brinkman,^{1*} J. L. Cashdollar,^{1*} S. M. Griffin,^{1*} B. R. McMinn,^{1*} E. R. Rhodes,^{1*} E.A. Varughese,^{1*} M. R. Karim,¹ A. G. Grimm,¹ S. K. Spencer,² and M. A. Borchardt²
U.S. Environmental Protection Agency, Office of Research and Development,
National Exposure Research Laboratory, Cincinnati, OH;¹ and USDA-Agricultural
Research Service,² Marshfield, WI

*NEB, JLC, SMG, BRM, ERR, and EAV contributed equally to the molecular method
development

DISCLAIMER

This method has been reviewed by the U.S. Environmental Protection Agency (EPA)'s Office of Research and Development (ORD) and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

ACKNOWLEDGEMENTS

The authors thank Drs. Sandhya Parshionikar, Keya Sen, and Carrie Miller from EPA's Office of Water, and Drs. George Di Giovanni from the Texas AgriLife Research Center, El Paso, TX; Paul Hazelton from the University of Manitoba, Winnipeg, Manitoba, Timothy Straub from the Pacific Northwest National Laboratory, Richland, WA, and Susan Boutros from Analytical Associates, Ithaca, NY for reviewing this manuscript and for providing helpful comments. Mohammad R. Karim was supported through an appointment to the Research Participation Program at ORD's National Exposure Research Laboratory, which was administered by the Oak Ridge Institute for Science and Education through an Interagency Agreement between the U.S. Department of Energy and EPA. The authors thank Dr. Skip Virgin of Washington University School of Medicine, St. Louis, MO for Murine norovirus.

Questions regarding this method or its application should be addressed to:

G. Shay Fout, Ph.D.
U.S. Environmental Protection Agency
Office of Research and Development
National Exposure Research Laboratory
Microbiological and Chemical Exposure
Assessment Research Division
Cincinnati, OH 45268-1320
(513) 569-7387
(513) 569-7117 (fax)
fout.shay@epa.gov

METHOD 1615. Enterovirus and Norovirus occurrence in water

ABBREVIATIONS USED

BGM	Buffalo Green monkey kidney cells
BSA	Bovine serum albumin
Cat. No.	Catalog number
cDNA	Complementary DNA
CL	Confidence limit
Ct	Cycle threshold
Cp	Crossing point
Cq	Quantitation point
CPE	Cytopathic effect
CV	Coefficient of variation
D	Volume of original water sample assayed
dH ₂ O	Distilled or deionized water
DNA	Deoxyribonucleic acid
EPA	United States Environmental Protection Agency
EV	Enteroviruses belonging to the genus, <i>Enterovirus</i>
FCSV	Final concentrated sample volume
GC	Genome copy
HGV	Hepatitis G virus
ICR	Information Collection Rule
LIMS	Laboratory information management system
MEM	Minimal essential medium
MPN	Most probable number
MSDS	Material Safety Data Sheet
Negative FCSV	Final concentrated sample volume from a negative QC sample
NoV GI	Genogroup I noroviruses belonging to the genus, <i>Norovirus</i>
NoV GII	Genogroup II noroviruses belonging to the genus, <i>Norovirus</i>
NPT	National pipe thread
NTU	Nephelometric Turbidity Units
ORD	Office of Research and Development
OSHA	Occupational Safety and Health Administration
OW	Office of Water
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Performance evaluation
QC	Quality control
qPCR	Quantitative polymerase chain reaction
RT-PCR	Reverse transcription-polymerase chain reaction
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
RNA	Ribonucleic acid
RPM	Revolutions per minute

METHOD 1615. Enterovirus and Norovirus occurrence in water

RT	Reverse transcription
S	Assay sample volume
SOP	Standard operating procedure
TCVA	Total cultural virus assay
TSV	Total sample volume
U.S.	United States

METHOD 1615. Enterovirus and Norovirus occurrence in water

TABLE OF CONTENTS

ABBREVIATIONS USED	iii
SCOPE AND APPLICATION	1
BACKGROUND	1
METHOD CONSTRAINTS	3
SUMMARY OF METHOD	3
DEFINITIONS	3
INTERFERENCES	6
SAFETY	7
EQUIPMENT AND SUPPLIES	8
REAGENTS, MEDIA, AND STANDARDS	12
QUALITY ASSURANCE	16
SAMPLE COLLECTION, PRESERVATION, AND STORAGE	20
SAMPLE COLLECTION	20
SHIPMENT OF SAMPLES	23
LABORATORY HOLDING TIME AND TEMPERATURE	24
FILTER ELUTION PROCEDURE	24
ELUTION EQUIPMENT SETUP	24
ELUTION	25
ORGANIC FLOCCULATION CONCENTRATION PROCEDURE	26
ORGANIC FLOCCULATION	26
RECONCENTRATED ELUATE	26
TOTAL CULTURABLE VIRUS ASSAY	29
QUANTAL ASSAY	29
VIRUS QUANTITATION	33
ENTEROVIRUS AND NOROVIRUS MOLECULAR ASSAY	34
PRELIMINARY PROCEDURES	35
TERTIARY CONCENTRATION	35
NUCLEIC ACID ISOLATION	36
REVERSE TRANSCRIPTION (RT)	37
REAL-TIME QUANTITATIVE PCR (qPCR)	38
METHOD PERFORMANCE	42
CULTURABLE ASSAY	42
MOLECULAR PROCEDURE	43
STERILIZATION AND DISINFECTION	44
TABLES AND FIGURES	47
DATA SHEETS	56
REFERENCES	62

METHOD 1615. Enterovirus and Norovirus occurrence in water

LIST OF TABLES

Table 1. Viruses Detected by EPA Method 1615	47
Table 2. Specified and Recommended Sample Volumes	47
Table 3. MPN Program Default Settings for MPN Calculator	48
Table 4. Primers and TaqMan [®] Probes for Virus Detection by RT-qPCR.....	48
Table 5. RT Master Mix 1 and 2.....	49
Table 6. PCR Master Mix for Enterovirus Assay	50
Table 7. PCR Master Mix for Norovirus GIA Assay	50
Table 8. PCR Master Mix for Norovirus GIB Assay.....	51
Table 9. PCR Master Mix for Norovirus GII Assay	51
Table 10. PCR Master Mix for Hepatitis G Assay	52
Table 11. Mean Recovery and Coefficient of Variation Range.....	52

LIST OF FIGURES

Figure 1. Uninfected BGM Cells	53
Figure 2. Early CPE from Poliovirus.....	53
Figure 3. Sample Filtration Apparatus.....	54
Figure 4. Elution of an Electropositive Filter with Beef Extract	54
Figure 5. RT-qPCR Schematic	55

METHOD 1615. Enterovirus and Norovirus occurrence in water

1. SCOPE AND APPLICATION

1.1. BACKGROUND

1.1.1. Introduction

- 1.1.1.1. EPA Method 1615 provides culture and molecular procedures for detecting human enteroviruses, human noroviruses, and mammalian orthoreoviruses (culture procedure only) in water (Table 1). The cell culture procedure detects enterovirus and orthoreovirus species that are capable of infecting and producing cytopathic effects (CPE) in the Buffalo Green monkey kidney (BGM) cell line (16, 19). Although this cell line is considered a “gold standard” for detection of infectious waterborne viruses, noroviruses and a number of enteroviruses do not replicate in BGM cells. There is no established cell line for detection of infectious human noroviruses, but a prototype research method is under development (37, 38). The molecular procedure incorporated into EPA Method 1615 detects the noroviruses and enteroviruses shown in Table 1, including those enteroviruses that do not replicate on BGM cells. However, it should be noted that a positive result indicates the presence of viral nucleic acid and not the infectivity status of the detected virus.
- 1.1.1.2. Enteroviruses and noroviruses are enteric viruses that replicate within the gastrointestinal tract and are spread through the fecal-oral route. They cause a variety of waterborne infections through exposure to contaminated drinking and recreational waters. Infections may be asymptomatic or result in mild gastroenteritis, febrile illness, or respiratory symptoms. They can also cause a variety of serious diseases such as aseptic meningitis, encephalitis, flaccid paralysis, hand, foot and mouth disease, hemorrhagic conjunctivitis, myocarditis, neonatal sepsis-like disease, or severe gastroenteritis (3, 20, 25). Enteroviruses and noroviruses have not only been found in drinking and recreational waters, but have caused waterborne outbreaks (4, 5, 18, 26, 27, 41). Due to public health concern, these viruses are on EPA’s Contaminant Candidate List 3 (<http://www.epa.gov/safewater/ccl/ccl3.html>). The *Mammalian orthoreovirus* species is not associated with any known waterborne outbreaks and usually does not cause disease in humans (13, 15). If desired, orthoreoviruses can be assayed using the molecular method found in Fout et al. (18).
- 1.1.1.3. Molecular procedures, such as polymerase chain reaction (PCR) and reverse transcription-PCR (RT-PCR), provide the flexibility to detect all waterborne human enteric viruses for which genome sequence data is available (18). The advent of

METHOD 1615. Enterovirus and Norovirus occurrence in water

real time quantitative PCR (qPCR) has resulted in additional advantages over conventional PCR in that quantitative results can be obtained in a very short time (21). These molecular methods have been widely used to detect viruses in environmental waters (7, 8, 22, 27, 28, 33, 39). Despite the advantages, molecular techniques are subject to three main limitations. First, PCR methods assay smaller volumes than culture methods, resulting in lower detection limits. Second, these methods are sensitive to inhibitors that are present in some environmental samples. To address this problem, controls are used to determine whether negative results are true negative or false negative values. Finally, molecular methods do not distinguish between infectious and noninfectious viruses. Therefore, a positive PCR assay for a particular pathogen in drinking water does not directly address issues of public health. Research is ongoing on several promising approaches to detect infectious viruses (31, 34). However, PCR is still a useful public health tool in spite of these problems. Because there is a strong relationship between indicator measurements by qPCR and health effects in recreational waters (40), EPA is considering using qPCR to set new criteria for monitoring recreational beaches. At the very least, positive PCR virus findings provide a warning of possible contamination issues, but recent studies have indicated a direct relationship between health effects and positive RT-qPCR findings for human viruses (Borchardt et al., manuscript in preparation).

- 1.1.2. Development of the ICR Total Culturable Virus Assay - In the 1990s EPA issued an Information Collection Rule (ICR; Federal Register 61:24353-24388) that required all drinking water utilities serving a population over 100,000 to monitor their source water for viruses monthly for a period of 18 months. The Rule also required that systems finding greater than one infectious enteric virus particle per liter of source water to monitor their finished water on a monthly basis. One of the purposes of the Rule was to obtain national data on virus levels in source waters to determine the adequacy of treatment requirements. To support the Rule, a virus monitoring protocol was developed by virologists at the EPA and modified to reflect consensus agreements from the scientific community and public comments to the draft rule (19). This standard ICR Total Culturable Virus method, along with quality assurance and laboratory approval procedures (<http://www.epa.gov/microbes/icrmicro.pdf>), was incorporated into the ICR by reference. The results of the ICR survey indicated that culturable viruses were present in 24% of the source waters throughout the nation. Since the end of the ICR, the ICR Total Culturable Virus method has continued to be used in the U.S. and in international settings for the detection of culturable viruses in surface, ground, and

METHOD 1615. Enterovirus and Norovirus occurrence in water

treated waters (14, 19, 24), but the high cost of collecting and analyzing virus samples has limited the method's widespread use.

- 1.1.3. Development of Method 1615 – In the past few years, an alternative sampling protocol that significantly reduces the cost of sampling has been found to be equivalent in performance to the ICR method (24). Method 1615 is a modification of the ICR protocol. It incorporates the alternative sampling procedure and reduces the number of required cell culture replicates required by the ICR protocol. It also includes a molecular procedure that is a modification of a method used to survey groundwaters for enteric viruses in Wisconsin (9, 27).

1.2. METHOD CONSTRAINTS

- 1.2.1. This method is for use by analysts skilled in virus concentration, elution, cell culture, and molecular techniques.
- 1.2.2. Analysts must not deviate from any of the procedures described in this method if the data is being generated to fulfill EPA regulatory requirements. For example, alternative procedures for elution, secondary and tertiary concentration, and analyses by culture and RT-qPCR must not be used without prior approval by EPA.

2. SUMMARY OF METHOD

Viruses that may be present in environmental or finished drinking waters are concentrated by passage through electropositive filters. Viruses are eluted from the filters with a beef extract reagent and concentrated using organic flocculation. A portion of the concentrated eluate is then inoculated onto replicate flasks of BGM cells to measure infectious viruses. Cultures are examined for the development of cytopathic effects for two weeks and then re-passaged onto fresh cultures for confirmation. Virus concentration in each sample is calculated in terms of the most probable number (MPN) of infectious units per liter using EPA's MPN calculator. For molecular assays, the concentrated eluate is concentrated again by centrifugal ultrafiltration. The RNA is extracted from the concentrate and tested for enterovirus and norovirus RNA using real time quantitative reverse transcription-polymerase chain reaction (RT-qPCR). Virus concentrations for the molecular assay are calculated in terms of genomic copies of viral RNA per liter based upon a standard curve.

3. DEFINITIONS

3.1. ANALYSIS BATCH

- 3.1.1. All virus samples processed by an analyst within one week's span shall be considered a "batch". A week is defined as a 7-day period.
- 3.1.2. Each sample result must be associated with a batch number.
- 3.1.3. A positive and negative Quality Control (QC) sample set must be processed with each batch.
 - 3.1.3.1. Failure to obtain both a positive value in the positive QC sample and a negative value in the negative QC sample results in failure of the entire batch.

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 3.1.3.2. Obtaining a positive value in the positive QC sample and a negative value in the negative QC sample results in acceptance of the data from the entire batch.

3.2. BUFFALO GREEN MONKEY KIDNEY (BGM) CELLS

This is a stable cell line of monkey kidney cells that were originally developed at the University of Buffalo for clinical isolation of enteroviruses and later adapted for use in detecting infectious viruses in environmental samples (16). BGM cells form a monolayer of cells when propagated in tissue culture vessels. Figure 1 is a micrograph of uninfected BGM cells growing as a monolayer.

3.3. CONTAMINANT CANDIDATE LIST (CCL)

A list of chemicals and microbial agents under consideration for regulatory action by EPA. The current list may be obtained from <http://water.epa.gov/scitech/drinkingwater/dws/ccl/>.

3.4. CYTOPATHIC EFFECT (CPE)

The degeneration of cells caused by virus replication. It often involves the complete disintegration of cells. It may also be identified through changes in cell morphology. However, care must be taken in the use of changes in cell morphology as evidence of CPE, because uninfected BGM cells change morphology during mitosis. True CPE is always progressive and can be rated on a 0-4 scale, with the values 0, 1, 2, 3, and 4 indicating that 0% (Figure 1), 25% (Figure 2), 50%, 75%, and 100% of the monolayer is showing CPE, respectively. Additional examples of CPE can be found in Malherbe and Strickland-Cholmley (29).

3.5. CYTOTOXICITY

Cytotoxicity is the development of CPE from toxic components in the matrix. Cytotoxicity can be distinguished from viral CPE by its early development after sample inoculation or by the failure to observe CPE in the second passage required by this method. Unlike CPE, which begins as small clusters of killed cells (see Figure 2) after two or more days of incubation, cytotoxicity usually develops uniformly in all inoculated cell culture replicates within 24 hours of inoculation.

3.6. DETECTION LIMIT

The number of virus particles or genome copy numbers that can be detected in a given volume by a method with 95% confidence.

3.7. ENTERIC VIRUSES

Viruses that primarily infect and replicate in the gastrointestinal tract are known as enteric viruses. These include enteroviruses, noroviruses, rotaviruses, hepatitis A virus, adenoviruses, and reoviruses, among others. Enteric viruses are capable of causing a wide range of illnesses such as gastroenteritis, paralysis, aseptic meningitis, respiratory illness, fevers, myocarditis, and neonatal enteroviral sepsis. Enteric viruses can be present in human and animal feces, which can contaminate recreational and drinking water sources.

METHOD 1615. Enterovirus and Norovirus occurrence in water

3.8. ENTEROVIRUS

Enteroviruses are a genus in the *Picornaviridae* family. These viruses are among the most common viruses infecting humans worldwide. Enteroviruses are small (approximately 30 nm), nonenveloped, single-stranded, positive sense RNA viruses with an icosahedral capsid. Traditionally, human enterovirus serotypes have been classified into echoviruses, coxsackieviruses group A and B, and polioviruses. Current taxonomy based on molecular typing divides human enteroviruses into four species, *Human enterovirus A*, *B*, *C*, and *D*.

3.9. GROWTH AND MAINTENANCE MEDIUM

Growth medium consisting of a 50/50 (v/v) mixture of Minimum Essential Medium (MEM) and Leibovitz's L-15 medium with antibiotics and 10% bovine serum is recommended. Maintenance medium consists of a 50/50 mixture of Eagle's minimum essential medium (MEM) and Leibovitz's L-15 medium with antibiotics and 2% bovine serum.

3.10. INOCULATION

Inoculation is the placement of concentrated water samples onto a monolayer of cells in a culture vessel for growing or replication of viruses in the cells.

3.11. MATERIAL SAFETY DATA SHEETS (MSDS)

These sheets contain written information provided by vendors concerning a chemical's toxicity, health hazards, physical properties, fire, and reactivity data, including storage, spill, and handling precautions.

3.12. MONOLAYER

A single confluent layer of cells covering the bottom of a tissue culture dish or flask (Figure 1).

3.13. NOROVIRUS

Noroviruses constitute a genus in the *Caliciviridae* family. The genus is divided into five genogroups (GI-GV) and 29 genetic clusters (42). Noroviruses are recognized as a leading cause of nonbacterial gastroenteritis in humans. Noroviruses are small (approximately 27 nm) and the genome possess a positive sense, single stranded RNA in a nonenveloped icosahedral capsid. Due to the absence of a standardized and validated infectivity assay for human noroviruses, the presence of noroviruses in environmental waters must be measured using molecular methods.

3.14. PERFORMANCE EVALUATION/PERFORMANCE TEST SAMPLE (PE)

A sample containing Sabin poliovirus type 3 at a concentration unknown to the analysts. PE samples are typically sent to all laboratories performing the method from a single outside source when performance evaluation is required by EPA. The purpose of the PE sample is to demonstrate that all analysts performing the method can meet minimum performance standards.

3.15. QUALITY CONTROL SAMPLE (QC)

METHOD 1615. Enterovirus and Norovirus occurrence in water

This is a sample containing Sabin poliovirus type 3 at a known concentration. QC samples will typically be sent to all laboratories performing the method from a single outside source when performance evaluation is required by EPA. The purposes of the QC sample are to give laboratories a standard sample for training new analysts and to give EPA and laboratory QA officials a tool to evaluate method performance for all laboratory analysts.

3.16. QUANTITATIVE CYCLE [(Cq), also called Cycle Threshold (Ct) or Crossing Point (Cp)]

The cycle at which the fluorescence of a quantitative PCR assay crosses the threshold that defines a positive reaction or at which the second derivative maximum is reached (10, 11).

3.17. QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR) and REVERSE TRANSCRIPTION-qPCR (RT-qPCR)

This is a procedure for quantitatively detecting the levels of specific DNA in a sample, or following reverse transcription [RT, e.g., the synthesis of complementary DNA (cDNA) from RNA], the levels of specific RNA (e.g., viral) in a sample.

3.18. REAGENT WATER

This is deionized or distilled reagent grade water (dH₂O) with a resistivity greater than 1 Siemens per meter (S/m), i.e., 1 megohms-cm at 25°C; if available, reagent grade water with a resistivity greater than 0.1 S/m (10 megohms-cm) is preferred (2).

3.19. STANDARD OPERATING PROCEDURE

A Standard Operating Procedure (SOP) is a set of written instructions that document a routine or repetitive activity followed by an organization. The development and use of SOPs are an integral part of a successful quality system as it provides individuals with the information to perform a job properly, and facilitates consistency in the quality and integrity of data. EPA guidance on developing SOPs can be obtained from www.epa.gov/quality/qs-docs/g6-final.pdf.

4. INTERFERENCES

4.1. REAGENTS

To minimize cross contamination, Analytical Reagent or ACS grade chemicals (unless specified otherwise) and reagent water should be used to prepare all media and reagents. It is recommended that water, media, and other reagent solutions be purchased from commercial sources and that tissue culture grade water be used for preparation of tissue culture media.

4.2. MATRIX INTERFERENCE

Matrix interferences may lead to false negative results and are caused by colloidal, suspended, or dissolved substances that are present in the water sample. Matrix interference can vary across different water sources and even across time in the same source.

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 4.2.1. Matrix interference due to colloidal or suspended solids may reduce the water volume that can be passed through the positively charged filters used in this method. Prefilters (item 6.1.5) or more than one electropositive filter must be used to overcome this type of interference.
- 4.2.2. Matrix interference may be identified by its effects on the culture or molecular assays. This may be expressed as the development of cytotoxicity in culture assays and or by inhibition of molecular assays.

4.3. OTHER INTERFERENCE

- 4.3.1. Failure to dechlorinate treated tap water samples during sampling or prolonged exposure to ambient temperatures during sample transportation or in the laboratory can lead to virus loss.
- 4.3.2. Inadequate disinfection of the sampling apparatus and contamination of reagents and supplies can lead to sample contamination. Inadequate disinfection of the sampling apparatus is identified using negative Quality Control samples (Section 8.4.3).
- 4.3.3. Inadequate physical separation and controlled workflow may lead to PCR interference due to false positive results from contamination. EPA's guidance for processing and handling these samples must be followed to minimize this interference (36).

5. SAFETY

5.1. SAFETY PLAN

The biohazard associated with, and the risk of infection from, human enteric viruses is high in this method because potentially infectious viruses are handled. This method does not purport to address all the safety issues associated with its use. Each laboratory is responsible for establishing a safety plan that addresses appropriate safety and health practices prior to using this method. In particular, laboratory staff must know and observe the safety procedures required in a microbiology laboratory that handles pathogenic organisms while preparing, using, and disposing of sample concentrates, reagents and materials, and while operating sterilization equipment. Minimum requirements have been published by the U.S. Department of Health and Human Services (1).

5.2. SHIPMENT OF SAMPLES

The samples collected using this method may be shipped as non-infectious materials, unless they are known to contain virus or other infectious materials. If they are known to contain infectious materials, laboratories are responsible for packaging and shipping them according to all Department of Transportation, Centers for Disease Control and Prevention, and State regulations.

5.3. CHEMICAL SAFETY

Each laboratory is responsible for the safe handling of the chemicals used in this method. The OSHA laboratory standards can be found on line at <http://www.osha.gov/SLTC/laboratories/standards.html>.

METHOD 1615. Enterovirus and Norovirus occurrence in water

6. EQUIPMENT AND SUPPLIES

References to specific brands or catalog numbers are included as examples only and do not imply endorsement of the product. These references do not preclude the use of other vendors or supplies. However, equivalent method performance should be demonstrated for substitutions of the electropositive filter, beef extract, and qPCR reagents. All equipment should be cleaned according to the manufacturers' recommendations. Disposable supplies should be used wherever possible to reduce the possibility of cross contamination. Note: Storage of samples and reagents at temperatures below 0°C must be done using manual defrost freezers.

6.1. SAMPLE FILTRATION APPARATUS

Figure 3 shows the Sample Filtration Apparatus which has been modified from that given in Fout et al. (19) for use with the NanoCeram[®] electropositive cartridge filter. The modification also increases the efficacy for disinfecting the apparatus. The sample filtration apparatus shown in Figure 1 is recommended, but that shown in Figure VIII-1 of Fout et al. (19) also may be used for virus monitoring. The apparatus may have to be modified using appropriate size housings and adapters for use with other equivalent filters, such as the ten-inch 1MDS Virosorb cartridge filter. The current configuration does not use a pressure regulator or pressure gauge, as these components are difficult to disinfect and subject to corrosion; however, laboratories are responsible for ensuring that water pressure at sampling sites does not exceed the pressure ratings of the cartridge housings used (125 psi for item 6.1.2.2).

6.1.1. Intake Module

- 6.1.1.1. Backflow regulator (Watts Regulator Series 8 C Hose Connection Vacuum Breaker; this component is optional)
- 6.1.1.2. Swivel female insert with garden hose threads (United States Plastic Cat. No. 63003)
- 6.1.1.3. ½" tubing (Cole-Parmer Cat. No. 06601-03) and hose clamps (Cole-Parmer Cat. No. 06403-10)
- 6.1.1.4. ½" hose barb quick disconnect body (Cole Parmer Cat. No. 31307-11)
- 6.1.1.5. Thread tape (Cole Parmer Cat. No. 08270-34)

6.1.2. Cartridge Housing Module for NanoCeram filters

- 6.1.2.1. ½" NPT (M) quick disconnect insert (Cole Parmer Cat. No. 31307-31; connected to the inlet port of the cartridge housing)
- 6.1.2.2. Cartridge housing (Argonide Cat. No. H2.5-5)
- 6.1.2.3. ½" NPT (M) quick disconnect body (Cole Parmer Cat. No. 31307-06; connected to the outlet port of the cartridge housing)
- 6.1.2.4. 5" NanoCeram cartridge filter (Argonide Cat. No. VS2.5-5).

6.1.3. Discharge Module

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 6.1.3.1. ½” NPT (M) quick disconnect insert (Cole Parmer Cat. No. 31307-31)
- 6.1.3.2. ½” NPT (F) straight connector (Cole Parmer Cat. No. 06349-03)
- 6.1.3.3. Flow meter (Flow Technology Cat. No. FT6-8NENWULEG-3)
– Note: the flow meter calibration at the flow rates used for sampling should be confirmed before use and after every month of use. The calibration can be performed by measuring the time to fill a 4 L or larger graduated cylinder (Cole Parmer Cat. No. 06135-90; e.g., 24± 1 second for the 10 liter per minute rate).
- 6.1.3.4. Rate/Totalizer (Flow Technology Cat. No. BR30-5-A-4)
- 6.1.3.5. ½” NPT (F) straight connector (Cole Parmer Cat. No. 06349-03)
- 6.1.3.6. ¾” NPT (M) × ½” NPT (M) reduction nipple (Cole Parmer Cat. No. 06349-87)
- 6.1.3.7. ¾” NPT (F) bronze globe valve (Cole Parmer Cat. No. 98675-09)
- 6.1.3.8. ¾” NPT (M) × GHT(M) fitting (United States Plastic Cat. No. 63016)
- 6.1.3.9. A garden hose of sufficient length to reach a drain.
- 6.1.4. Injector module – this module, prepared using the components below, should only be used when it is necessary to add thiosulfate or other additives to water during sampling. It can be modified into a double injector module for the addition of more than one additive by installing a small ¼” NPT (F) TEE (Cole Parmer Cat. No. 06349-51) on top of the larger TEE (item 6.1.4.2) via the male reducer (item 6.1.4.4) and placing items 6.1.4.5 and 6.1.4.6 on each end of the smaller TEE using a ¼” NPT (M) Nipple (Cole Parmer Cat No. 30623-25).
 - 6.1.4.1. ⅜” NPT (M) quick disconnect insert (Cole Parmer Cat. No. 31307-30, attached to the left port of the TEE)
 - 6.1.4.2. ⅜” NPT (F) TEE (Cole Parmer Cat. No. 06482-84)
 - 6.1.4.3. ⅜” NPT (M) quick disconnect body (Cole Parmer Cat. No. 31307-05, attached to the right port of the TEE)
 - 6.1.4.4. ⅜” NPT (M) × ¼” NPT (M) male reducer (Cole Parmer Cat. No. 30623-42; this and following components are connected to the top port of the TEE)
 - 6.1.4.5. ¼” NPT (F) metallic check valve (CV; Cole Parmer Cat. No. 98676-00)

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 6.1.4.6. $\frac{1}{4}$ " NPT (M) \times $\frac{1}{4}$ " tubing ID male pipe adaptor elbow (Cole Parmer Cat. No. 30622-97)
- 6.1.4.7. 15-gallon chemical tank (Grainger Cat. No. 4UP09) with tubing. This item is for injecting 2% thiosulfate (item 7.3) into water containing a disinfectant. The container size can be adjusted to meet the anticipated need.
- 6.1.4.8. Chemical metering pump (Grainger Cat. No. 4UP03; before first use, adjust the chemical metering pump to deliver 2.4 or 6 mL/minute (i.e., 0.6 mL \times L of disinfected water passing through the sample filtration apparatus each minute) for flow rates of 4 or 10 L/minute, respectively. Using a graduated cylinder to measure the flow rate, and record or mark the pump setting for each rate.
- 6.1.5. Prefilter module – for use with waters exceeding 75 nephelometric turbidity units (NTU) or for other conditions that prevent the minimum sampling volumes from being obtained. Note: The NanoCeram filter is more susceptible to clogging than the 1MDS filter. A prefilter module may be required for some matrices even when the turbidity is considerably below 75 NTU.
 - 6.1.5.1. Prepare the prefilter module as described step 6.1.2, except use the filter described in step 6.1.5.2
 - 6.1.5.2. 10 μ m Polypropylene Prefilter Cartridge (Parker Hannifin Cat. No. M19R5-A)
- 6.1.6. Assemble the modules as shown in Figure 3 using thread tape (item 6.1.1.5) on all connections. Sterilize the regulator, prefilter housing, and cartridge housings with chlorine as described in Section 15. Using aseptic technique, add a sterile NanoCeram or 1MDS cartridge to the cartridge housing and, if needed, a presterilized polypropylene cartridge to the prefilter housing. Cover the ends with sterile aluminum foil.

6.2. OTHER SAMPLING AND SHIPPING MATERIALS

- 6.2.1. Peristaltic or chemical resistant pump capable of pumping water at 4-10 liters/min and appropriate connectors (for use where garden hose-type pressurized taps for the source or finished water to be monitored are unavailable and for QC samples). Follow the manufacturer's recommendations for pump priming.
- 6.2.2. One L polypropylene wide-mouth bottles (Nalgene Cat. No. 2104-0032)
- 6.2.3. Collapsible 10 L LDPE cubitainer (Cole Parmer Cat. No. 06100-30)
- 6.2.4. Portable pH and temperature probe (Omega Cat. No. PHH-830)
- 6.2.5. Portable turbidity meter (Omega Cat. No. TRB-2020)
- 6.2.6. Portable Chlorine (Free & Total), Pocket Colorimeter II Test Kit with reagents (Hach Cat. No. 5870062).

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 6.2.7. Commercial ice packs (Cole-Parmer Cat. No. 06345-20)
- 6.2.8. iButtons (Maxim Cat. No. DS1921G)
- 6.2.9. Insulated shipping box with carrying strap (17" × 17" × 16"; Cole-Parmer Cat. No. 03742-00 and 03742-30) or insulated storage and transport chest (Fisher Scientific Cat. No. 39)
- 6.2.10. Miscellaneous - aluminum foil, surgical gloves, waterproof marker

6.3. EQUIPMENT AND SUPPLIES FOR THE ORGANIC FLOCCULATION PROCEDURE

- 6.3.1. Dispensing pressure vessels, 5 and 20 L capacity (Millipore Corp. Cat. No. XX6700P05 and XX6700P20)
- 6.3.2. Pressure source - laboratory positive pressure air line (equipped with oil filter), compressed nitrogen, peristaltic pump (e.g., Cole Parmer Cat. No. 07523-60), or self-priming pump (e.g., Cole Parmer Cat. No. 07036-10)
- 6.3.3. pH meter with combination-type electrode and an accuracy of at least 0.1 pH units
- 6.3.4. Magnetic stirrer and stir bars
- 6.3.5. Refrigerator, set at 4°C ± 3°C
- 6.3.6. Refrigerated centrifuge capable of attaining 4,000 ×g
 - 6.3.6.1. Screw-capped centrifuge bottles with 100 to 1000 mL capacity. Each bottle must be rated for the relative centrifugal force used.
 - 6.3.6.2. Appropriate centrifuge buckets for centrifuge bottles
- 6.3.7. Sterilizing filter – 0.22 µm Acrodisc filter with prefilter (VWR Cat. No. 28143-295) for use at step 11.2.3.8.
- 6.3.8. 47 mm disc filter holder (Millipore Corp. Product No. SX0004700)
- 6.3.9. Sterilizing filter stack – Place a 0.22 µm pore-size membrane filter (Millipore Corp. Product No. GSWP04700) on the bottom of a 47 mm disc filter holder.
 - 6.3.9.1. Place an AP15 prefilter (Millipore Corp. AP1504700) on top of the 0.22 µm filter and an AP20 prefilter (Millipore Corp. AP2004700) on top of the AP15 prefilter.
 - 6.3.9.2. Assemble the filter holder unit and sterilize as defined in section 15.2.2.
 - 6.3.9.3. Note: Disassemble the filter stack after each use to check the integrity of the 0.22 µm filter. Refilter any media filtered with a damaged stack using another sterile sterilizing filter stack.
 - 6.3.9.4. Note: The sterilizing filter stack is optional, but should be used for samples that are difficult to filter using item 6.3.7.

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 6.3.10. Sterilizing filter – 0.22 μm (Corning Cat. No. 431219) for use at step 12.1.4.2.

6.4. EQUIPMENT AND SUPPLIES FOR THE TOTAL CULTURABLE VIRUS ASSAY

- 6.4.1. Mechanical rocking platform (Daigger Product No. EF4907G)
- 6.4.2. Incubator capable of maintaining the temperature of cell cultures at $36.5 \pm 1^\circ\text{C}$
- 6.4.3. Flasks or bottles for BGM culture maintenance and quantal assays (e.g., 2100 cm^2 roller bottles, Fisher Cat. No. 73520-420; 850 cm^2 roller bottles, Corning Cat. No. 430849; 25 cm^2 flasks, Sigma Cat. No. C6231)

6.5. EQUIPMENT FOR THE TERTIARY CONCENTRATION PROCEDURE AND THE MOLECULAR ASSAYS

- 6.5.1. UV-Vis Spectrophotometer (Thermo Fisher Scientific Cat. No. NanoDrop 2000)
- 6.5.2. Vivaspin 20 – 30,000 MWCO (Sartorius-Stedim Cat. No. VS2022; other centrifugal concentrators with 30,000 MWCO may be substituted for this item, if equivalent recoveries are demonstrated)
- 6.5.3. 50 mL polypropylene centrifuge tube and appropriate buckets for centrifuge (item 6.3.6)
- 6.5.4. Microcentrifuge capable of 20,000 $\times g$ (Fisher Scientific Cat. No. 05-406-11)
- 6.5.5. 1.5 mL microcentrifuge tubes (Fisher Scientific Cat. No. 02-682-550)
- 6.5.6. Vortex mixer (Fisher Scientific Cat. No. 02-216-100)
- 6.5.7. Thermal cycler (Applied Biosystems Cat. No. 4314879)
- 6.5.8. Optical reaction plate (Applied Biosystems Cat. No. 4314320)
- 6.5.9. Real-Time PCR system (Applied Biosystems Cat. No. 4351405)

7. REAGENTS, MEDIA, AND STANDARDS

The amount of reagents, media, and standards prepared for each step of the method may be adjusted proportionally to the number of samples to be analyzed. For any given section of this method only media, reagents, and standards that are not described in previous sections are listed.

7.1. REAGENTS FOR THE SAMPLING PROCEDURE

- 7.1.1. Hydrochloric acid (HCl) – Prepare 0.12, 1.2, and 6 M solutions by mixing 50, 100, or 50 mL of concentrated HCl (Note: HCl at 37% concentration is about 12 M) with 4950, 900, or 50 mL of dH_2O , respectively. Prepare solutions to be used for adjusting the pH of water samples at least 24 h before use. HCl solutions can be stored for several months at room temperature.

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 7.1.2. QC stock – This is a stock of Sabin poliovirus type 3 containing 200 MPN/mL. This stock may be prepared by the analytical laboratory or, if available, obtained from a contractor designated by the US EPA.
- 7.1.3. Matrix spike – This is a stock of Sabin poliovirus type 3 containing 1000 MPN/mL. This stock may be prepared by the analytical laboratory or, if available, obtained from a contractor designated by the US EPA.
- 7.1.4. Sodium hypochlorite (NaClO) – Prepare a 0.525% NaClO solution by diluting household bleach 1:10 in water. 0.525% NaClO solutions can be stored for one week at room temperature.
- 7.1.5. 1 M sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) pentahydrate – Prepare a 1 M solution by dissolving 248.2 g of $\text{Na}_2\text{S}_2\text{O}_3$ in 1 liter of dH_2O . Sodium thiosulfate solutions may be stored for six months at room temperature.
- 7.1.6. 2 % sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) pentahydrate – Prepare 2% thiosulfate by dissolving 1 kg of sodium thiosulfate pentahydrate into 49 L of sterile water. Sodium thiosulfate solutions may be stored for six months at room temperature.

7.2. REAGENTS FOR THE ELUTION AND ORGANIC FLOCCULATION PROCEDURES

- 7.2.1. Sodium hydroxide (NaOH) – Prepare 0.1, 1, and 5 M solutions by dissolving 0.4, 4, or 20 g of NaOH in a final volume of 100 mL of dH_2O , respectively. NaOH solutions may be stored for several months at room temperature.
- 7.2.2. 0.5% iodine – Prepare a 0.5% iodine solution by dissolving 5 g of iodine in 1 L of 70% ethanol. Iodine solutions can be stored for one year at room temperature.
- 7.2.3. 1.5% beef extract, pH 9.0
 - 7.2.3.1. Prepare buffered 1.5% beef extract by dissolving 30 g of beef extract, desiccated (BD Bacto Cat. No. 211520) powder and 7.5 g of glycine (final glycine concentration = 0.05 M) in 1.9 L of dH_2O .
 - 7.2.3.2. Adjust the pH to 9.0 with 1 or 5 M NaOH and bring the final volume to 2 L with dH_2O .
 - 7.2.3.3. Autoclave the beef extract solution at 121°C for 15 minutes and use at room temperature. Beef extract solutions may be stored overnight at room temperature or 4°C, for one week at 4°C, or for longer periods at -20°C.
 - 7.2.3.4. Warning: Desiccated beef extract lots show considerable variation in virus recovery. Therefore, each new lot of beef extract must be screened before use in the Organic Flocculation Concentration Procedure to determine whether virus recoveries are adequate. Perform the screening by spiking one liter of

METHOD 1615. Enterovirus and Norovirus occurrence in water

beef extract solution with 1 mL of a QC sample. Process the spiked sample according to the Organic Flocculation and Total Culturable Virus Assay procedures given below. The mean recovery of poliovirus for three trials should be greater than 50%.

- 7.2.4. 1.5% beef extract, pH 7.0 – 7.5 - Prepare 1.5% beef extract by dissolving 7.5 g of beef extract, desiccated powder and 1.88 g of glycine in 0.5 L of dH₂O. Autoclave the beef extract solution at 121°C for 15 minutes and use at room temperature. This beef extract solution may be stored for up to six months at room temperature, but must be discarded if there is evidence of microbial growth or any other change in appearance.
- 7.2.5. 0.15 M sodium phosphate, pH 9.0 – Prepare 0.15M sodium phosphate by dissolving 40.2 g of sodium phosphate, dibasic (Na₂HPO₄ • 7H₂O) in a final volume of 1000 mL dH₂O. Adjust the pH to 9.0 with HCl, if necessary. Autoclave at 121°C for 15 minutes. Sodium phosphate solutions (items 7.9 and 7.10) may be stored at room temperature for up to 12 months.
- 7.2.6. 0.15 M sodium phosphate, pH 7.0-7.5 – Prepare by dissolving 40.2 g of sodium phosphate, dibasic (Na₂HPO₄ • 7H₂O) in a final volume of 1000 mL dH₂O. Adjust the pH to 7.0 to 7.5 with HCl. Autoclave at 121°C for 15 minutes.
- 7.2.7. Antifoam (Sigma Cat. No. A8311)

7.3. REAGENTS FOR THE TOTAL CULTURABLE VIRUS ASSAY

- 7.3.1. Cell culture test vessels containing confluent monolayers of BGM cells – BGM cells between passages 117 and 250 must be used. Cells should be passaged and maintained using the standard procedures available in the most recent version of the USEPA Manual of Methods for Virology (6), available at <http://www.epa.gov/microbes/about.html>. BGM cells from various sources and other standard tissue culture techniques and media may be used as long as analysts meet the acceptance criteria listed in section 14. Cell cultures used for virus assay are generally found to be at their most sensitive level between the third and sixth days after their most recent passage; those older than seven days must not be used.
- 7.3.2. Cell culture media
 - 7.3.2.1. Hank's balanced salt solution (Invitrogen Cat. No. 14170-112)
 - 7.3.2.2. Fetal bovine serum, certified, heat-inactivated (Invitrogen Cat. No. 10082-139)
 - 7.3.2.3. Trypsin with EDTA (Invitrogen Cat. No. 25300-062)
 - 7.3.2.4. Fungizone (Invitrogen Cat. No. 15290-018), Penicillin-Streptomycin (Invitrogen Cat. No. 15140-122)

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 7.3.2.5. Minimum essential medium (MEM) with Hanks' salts and L-glutamine (Sigma Aldrich Cat. No. M1018)
- 7.3.2.6. Leibovitz's L-15 medium with L-glutamine (Sigma Aldrich Cat. No. L4386).
- 7.3.2.7. 7.5% Sodium bicarbonate (Sigma Aldrich Cat. No. S8761)
- 7.3.2.8. Trypan blue (Sigma-Aldrich Cat. No. T8154)
- 7.3.3. Positive Assay Control – Prepare by diluting the QC stock in sodium phosphate, pH 7.0 - 7.5, to give a concentration of 20 PFU per Inoculation Volume (see step 11.2.4.5 for a definition of Inoculum Volume).

7.4. REAGENTS FOR THE TERTIARY CONCENTRATION PROCEDURE AND THE MOLECULAR ASSAYS

- 7.4.1. Dulbecco's Phosphate Buffered Saline, without CaCl_2 and MgCl_2 (U.S Biological Cat. No. D9820)
- 7.4.2. BSA, crystalline (United States Biochemical Cat. No. 10856)
- 7.4.3. 0.2 μm sterilizing filter (Sigma Cat. No. F-9768)
- 7.4.4. 5% BSA – Prepare 5% BSA by dissolving 5 g of in 100 mL of dH_2O . Sterilize by passing the solution through a sterilizing filter (item 7.4.3). Store at 4°C .
- 7.4.5. PBS, 0.2% BSA – Prepare by adding 4 mL of 5% BSA to 96 mL of PBS. Sterilize by passing the solution through a 0.2 μm sterilizing. Store at 4°C .
- 7.4.6. QIAamp DNA Blood Mini Kit (Qiagen Cat. No. 51104 or 51106)
- 7.4.7. Buffer AVL (Qiagen Cat. No. 19073; carrier RNA is supplied with this reagent)
- 7.4.8. Buffer AVE (Qiagen Cat. No. 1026956)
- 7.4.9. Collection tubes, 2 mL (Qiagen Cat. No. 19201)
- 7.4.10. Absolute ethanol (Fisher Scientific Cat. No. BP2818-100)
- 7.4.11. Random Primers, 0.5 $\mu\text{g}/\mu\text{L}$ (Promega Cat. No. C1181)
- 7.4.12. 10X PCR Buffer II with 25 mM MgCl_2 (Applied Biosystems Cat. No. N808130)
- 7.4.13. Deoxyribonucleotides, 10 mM (NTPs; Promega Cat. No. C1141)
- 7.4.14. Dithiothreitol, 100 mM (DTT; Promega Cat. No. P1171)
- 7.4.15. RNasin® Plus RNase Inhibitor, 40 units/ μL (Promega Cat. No. N2615)
- 7.4.16. SuperScript II Reverse Transcriptase, 200 units/ μL (Invitrogen Cat. No. 18064)
- 7.4.17. LightCycler 480 Probes Master kit (Roche Diagnostics Cat. No. 04707494001)

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 7.4.18. ROX reference dye, 25 mM (Invitrogen Cat. No. 12223)
- 7.4.19. Hepatitis G virus Armored RNA[®] (Asuragen Cat. No. 42024)
- 7.4.20. Enterovirus, norovirus GI, norovirus GII Armored RNA (Asuragen custom order giving $>10^{10}$ genomic copies at a defined concentration)

8. QUALITY ASSURANCE

This section describes the minimum quality assurance (QA) requirements. Laboratories are encouraged to institute additional QC practices that go beyond these minimum criteria to meet their needs.

8.1. QUALITY ASSURANCE PLAN

All laboratories analyzing samples with this method must adhere to defined quality assurance procedures that ensure analytical data which are scientifically valid and which demonstrate acceptable precision and specificity. Each laboratory must have a written Quality Assurance Plan that addresses the following:

- 8.1.1. Laboratory organization and responsibility – This section must include the following: 1) a list that identifies the laboratory QA manager(s) and key individuals who are responsible for ensuring the production of valid measurements and the routine assessment of QC data, 2) specify who is responsible for internal audits and reviews of the implementation of the QA plan and its requirements, and 3) include a chart showing the laboratory organization and line authority.
- 8.1.2. Personnel – This section must list each analyst's academic background and experience, describe how each analyst is trained to perform the method, and describe how training is documented.
- 8.1.3. Facilities – This section must describe the arrangement and size of laboratories, workflow patterns to minimize cross contamination, air system; laboratory reagent water system, and waste disposal system [see reference (36)].
- 8.1.4. Field sampling procedures – This section must describe the laboratory chain-of-custody procedures, including the sample identification and information recording system; and describe how field samples are collected and transported, including transportation time and temperature.
- 8.1.5. Laboratory sample handling procedures – This section must describe sample-holding times and temperature during analyses and the procedures for maintaining the integrity of the samples, i.e., logging and tracking of samples from receipt through analyses and disposal.
- 8.1.6. Equipment – This section must describe the specifications, calibration procedures, preventive maintenance, and how quality control records are maintained for each item used during the performance of the method. All calibrations must be traceable to national standards, when they are available.

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 8.1.7. Supplies – This section must describe the specifications, storage conditions, and how catalog and lot numbers are documented for chemicals, reagents, and media.
- 8.1.8. Laboratory practices – This section must describe the preparation of reagent-grade water, glassware washing and preparation procedures, and sterilization procedures. It should also describe the workflow requirements among laboratories to prevent cross contamination, especially for molecular procedures. The work flow and other recommended requirements are described in detail in Sen et al. (36).
- 8.1.9. Analytical procedures – This section must reference this method and identify available laboratory SOPs.
- 8.1.10. Quality control checks – This section must describe all laboratory procedures that are implemented to ensure the quality of each analyst's data.
- 8.1.11. Data reduction, verification, and reporting – This section must describe any procedures for converting raw to final data, identify procedures for ensuring the accuracy of data transcription and calculations, and describe the laboratory's procedures for reporting all data to EPA.
- 8.1.12. Corrective actions – This section must describe how the laboratory will respond to PE and QC failures and from failures of its own internal QC procedures, identify the person(s) responsible for taking corrective action, and describe how the effectiveness of the actions will be documented.
- 8.1.13. Record keeping – This section must describe how records are maintained (e.g., hard copy, electronic or LIMS, etc.), how long records are kept, and where records are stored.

8.2. LABORATORY PERSONNEL

- 8.2.1. Principal Analyst/Supervisor – Laboratories must have a principal analyst who may also serve as a supervisor if an additional analyst(s) is to be involved. The principal analyst/supervisor oversees or performs the entire analyses and carries out QC performance checks to evaluate the quality of work performed by analysts and technicians. This person must be an experienced microbiologist with at least a B.A./B.S. degree in microbiology or a closely related field. The person must also have a minimum of three years continuous bench experience in cell culture propagation, processing and analysis of virus samples, and in performing PCR, and at least six months of experience in performing RT-qPCR. This analyst must have analyzed a PE sample set and results must fall within acceptance limits. The principal analyst must also demonstrate acceptable performance during any on-site performance audits.
- 8.2.2. Analyst – The analyst performs at the bench level under the supervision of a principal analyst and can be involved in all aspects of analysis, including preparation of sampling equipment, filter extraction, sample processing, cell culture, virus assay, qPCR, and data handling. The analyst must have

METHOD 1615. Enterovirus and Norovirus occurrence in water

two years of college lecture and laboratory course work in microbiology or a closely related field. The analyst must have at least six months bench experience in cell culture, animal virus analyses and PCR, including three months experience in filter extraction of virus samples and sample processing. Six months of additional bench experience in the above areas may be substituted for the two years of college. Each analyst must have analyzed a PE sample set and results must fall within acceptance limits. The analyst must also demonstrate acceptable performance during any on-site audits. Should laboratories choose to use teams of analysts who specialize in performing the culture or molecular portions of this method, analysts only need to meet the educational requirement of the portion they perform. Laboratories using analyst teams must ensure that the PE samples are analyzed by the appropriate team member.

- 8.2.3. Technician – The technician extracts filters, processes samples, and performs qPCR under the supervision of an analyst, but does not perform cell culture work, virus detection, or enumeration. The technician must have at least three months experience in filter extraction, processing of virus samples to participate in the cultural portion of this method and three months of experience with PCR to participate in the molecular portion of the method.
- 8.2.4. Samplers – The sampler collects water samples and ships them to the analytical laboratory. The sampler must be familiar with the sample collection process and have at least training by means of a video or written instructions demonstrating proper sampling technique. Laboratories are responsible for ensuring that samplers have adequate training.

8.3. LABORATORY PERFORMANCE

- 8.3.1. Laboratories using this method must evaluate the ability of analysts to perform the method using known quality control (QC) and unknown performance evaluation (PE) samples as defined in Section 8.3.1.2 and 8.3.1.3. EPA may also require laboratories to be approved.
 - 8.3.1.1. Laboratory approval – Laboratories must have a Quality Assurance Plan, adequately trained staff, proper equipment, and at least one approved analyst to be approved.
 - 8.3.1.2. Initial analyst approval/initial demonstration of capability – Each analyst must demonstrate the ability to perform the method using QC and PE samples as part of an initial approval process. New analysts must initially use QC samples to gain method proficiency followed by the analysis of a PE sample set.
 - 8.3.1.3. Ongoing analyst approval/ongoing demonstration of capability – Each analyst must analyze one QC sample set for every analysis batch (see section 3) and one PE sample set per month following initial approval to remain approved. A QC sample set is comprised of a negative and a positive QC sample (see

METHOD 1615. Enterovirus and Norovirus occurrence in water

section 8.4). A PE sample set is comprised of two PE samples that may or may not contain virus at various levels (section 8.5). For initial and ongoing approval, analysts must meet the method performance characteristics defined in section 14 or in any additional guidance from EPA. Any analyst who does not meet the initial or ongoing demonstration of capability must not process samples until the cause of the failure has been identified and corrected.

8.4. QC SAMPLE SET

A QC sample set consists of a negative and a positive QC sample. QC stocks (item 7.1.2) with a titer of 200 MPN/mL should be prepared by the analytical laboratories and stored in aliquots containing about 1.1 mL at -70°C. QC sample results must meet the method performance characteristics defined in section 14.

- 8.4.1. Note: It is difficult to obtain an accurate pH on pure water. To compensate, a buffering agent, such as HEPES (Sigma Aldrich Cat. No. H4034), may be added to the water at a concentration up to 0.01M.
- 8.4.2. Note: QC samples should use the filter type (e.g., 1MDS or NanoCeram) that will be used for collecting samples. If both filter types are being used, the QC samples should be performed using the filter type that is most frequently analyzed by the analyst.
- 8.4.3. Negative QC Sample/Equipment blanks
 - 8.4.3.1. Place 10 L of reagent grade water in a dispensing pressure vessel or polypropylene container (e.g., Cole Parmer Product No. EW-06317-53). Adjust the pH to 6.5-7.5 with 0.12 M HCl or 0.1 M NaOH, as necessary.
 - 8.4.3.2. Place a magnetic stir bar into the vessel or container and stir for 10 minutes at a speed sufficient to create a vortex.
 - 8.4.3.3. Pass the water through a sterile standard filter apparatus (Section 6.1) containing a sterile electropositive filter using a flow rate of approximately 10 L/minute. Note: to meet ongoing QC requirements standard filter apparatuses that have been sterilized after use in the field must be used.
 - 8.4.3.4. Process and analyze the filter using the Filter Elution (section 10), Organic Flocculation (section 11), Total Culturable Virus Assay (section 12) and Enterovirus and Norovirus Molecular (section 13) procedures.
- 8.4.4. Positive QC Sample
 - 8.4.4.1. Place 10 L of reagent grade water in a dispensing pressure vessel or polypropylene container (e.g., Cole Parmer Product No. EW-06317-53). Adjust the pH to 6.5-7.5 with 0.12 M HCl or 0.1 M NaOH, as necessary.

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 8.4.4.2. Add 1.0 mL of a QC sample (see item 7.1.2) to the water.
- 8.4.4.3. Place a magnet into the vessel or container and stir for 10 minutes at a speed sufficient to create a vortex.
- 8.4.4.4. Pass the water through a sterile standard apparatus containing a sterile electropositive filter using a flow rate of approximately 10 L/minute.
- 8.4.4.5. Process and analyze the filter using the Elution (step 10), Organic Flocculation (step 11), Total Culturable Virus Assay (step 12) and Enterovirus and Norovirus Molecular (step 13) procedures.
- 8.4.5. QC sample stocks (item 7.1.2) are also to be used for the Positive Assay Control (see item 7.3.3 and step 12.1.2.3.2).

8.5. PE SAMPLES

- 8.5.1. PE samples will be sent to analysts in a randomized fashion and may contain no, low, medium, or high levels of Sabin poliovirus type 3 on the filter type (e.g., 1MDS or NanoCeram) used for sampling.
- 8.5.2. Process and analyze the PE filter using the Elution (section 10), Organic Flocculation (section 11), Total Culturable Virus Assay (section 12) and Enterovirus and Norovirus Molecular (section 13) procedures and according to any additional requirements supplied with the samples.
- 8.5.3. PE sample results should meet the method performance characteristics defined in section 14.

8.6. MATRIX SPIKE

Matrix spike should be run for every sample location initially and then after every 20th sample from the same location. Matrix spikes duplicates are performed by collecting two samples at the sampling location as described in section 9, except that the sampling volume of the second sample is reduced by 10 L. The last 10 L is collected in a 10 L cubitainer (item 6.2.3), shipped back to the laboratory, seeded with 1 mL of the matrix spike (item 7.1.3), passed through the duplicate filter, and analyzed by the method procedures (steps 10 through 12.2.11). The results of the analysis of the matrix spike must meet the performance measures in section 14.

8.7. RECORD MAINTENANCE

Laboratories shall maintain all records related to data quality. This shall include a record of the analyst name, date, and results of all QA controls performed, records of equipment calibration and maintenance, and reagent and material catalog and lot numbers used for all analytical procedures.

9. SAMPLE COLLECTION, PRESERVATION, AND STORAGE

9.1. SAMPLE COLLECTION

- 9.1.1. Filter sampling apparatus sterilization

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 9.1.1.1. Before each use, analytical laboratories must wash, and then sterilize the intake and cartridge housing modules, any necessary injector modules, and pumps as described in section 15.2.4.
- 9.1.1.2. Cover the apparatus module ends and the injector port(s) with sterile aluminum foil.
- 9.1.1.3. Place the injector module and tubing into a sterile bag or wrapping in such a way that they may be removed without contaminating them.
- 9.1.1.4. Ship the filter sampling apparatus components to the individuals who will be collecting water samples.
- 9.1.2. Preparation for sample collection
- 9.1.3. Note: Individuals collecting water samples for virus analysis must wear surgical gloves and avoid conditions that can contaminate a sample with virus. Gloves should be changed after touching human skin or handling components that may be contaminated (e.g., water taps, other environmental surfaces). Care must be taken to ensure that cartridge filters are properly seated in the housings. Housings with properly seated filters must not leak. Filters should be checked for proper seating upon opening the housing at the analytical laboratory by examining the gaskets for depressions that do not extend beyond the edge of the filter.]
 - 9.1.3.1. Purge the water tap to be sampled before connecting the filter apparatus. Continue purging for 2-3 minutes or until any debris that has settled in the line has cleared.
 - 9.1.3.2. Remove the foil from the backflow regulator. Loosen the swivel female insert slightly to allow it to turn freely and connect the backflow regulator to the tap. Retighten the swivel female insert. Disconnect the cartridge housing module at the quick connect, if connected, and cover the open end with sterile foil.
 - 9.1.3.3. Remove the foil from the ends of the discharge module and connect it to the regulator module. Place the end of the regulator module or the tubing connected to the outlet of the regulator module into a 1 L plastic bottle.
 - 9.1.3.4. Slowly turn on the tap and adjust the globe valve until the flow meter/totalizer reads 10 L/min. If the tap is incapable of reaching this flow rate, adjust the valve to achieve the maximum flow rate. Slower flow rates will result in longer sampling times.
 - 9.1.3.5. Flush the apparatus assembly with at least 75 L of the water to be sampled. While the system is being flushed, measure the

METHOD 1615. Enterovirus and Norovirus occurrence in water

chlorine residual, pH, temperature, and the turbidity of the water collecting in and overflowing from the 1 L plastic bottle.

- 9.1.3.6. Record the pH, temperature, and turbidity values onto a sample data sheet.
- 9.1.3.7. Turn off the water at the tap.
- 9.1.4. Injector module adjustment (Note: If a NanoCeram filter is being used and if the water pH is 9.0 or less and if it does not contain a disinfectant, skip to section 9.1.5. If disinfected waters above pH 8.0 are being used with a 1MDS cartridge filter, substitute a double injector module for the single injector module in the following steps. With 1MDS filters under these conditions, use a second metering pump connected to the second connection on the double injector module to add 0.12 M HCl at a rate, which brings the pH of the water exiting the discharge module to 6.5 to 7.5.)
 - 9.1.4.1. If the sample contains a disinfectant, turn off the water at the tap and disconnect the discharge module from the regulator module.
 - 9.1.4.2. Remove the foil from the ends of an injector module and connect the module to the quick connect of the regulator module.
 - 9.1.4.3. Turn on the metering pump. Set the pump to deliver 2.4 or 6 ± 0.2 mL/minute for flow rates of 4 or 10 L/minute, respectively (see Table 2). Measure the flow exiting the injector module for several minutes to ensure that the flow rate is correct. Measure the chlorine residual and if present, re-adjust the flow rate until no residual is present. Re-mark the setting, if necessary. Turn off the metering pump.
- 9.1.5. Virus collection
 - 9.1.5.1. If connected, remove the discharge module. Remove the foil from the cartridge housing module and connect it to the end of the regulator module, or if used, the injector module. Connect the discharge module to the outlet of the cartridge housing module.
 - 9.1.5.2. If the water sample has turbidity greater than 75 NTU, remove the foil from each end of the prefilter module and connect the prefilter module between the regulator module or, if used, the injector module and the cartridge housing module.
 - 9.1.5.3. Record the unique sample number, location, date, time of day and initial totalizer reading onto a sample data sheet (section 17.1).
 - 9.1.5.4. If an injector module is being used, turn on the metering pump.

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 9.1.5.5. With the filter housing placed in an upright position, slowly open the water tap until it is completely open (Note: If the cartridge housing has a vent button, press it while opening the tap to expel air from the housing. When the air is totally expelled from the housing, release the button, and open the sample tap completely. If the housing does not have a vent button, allow the housing to fill with water before completely opening the tap).
 - 9.1.5.5.1. After the tap is opened completely, check the flow rate and readjust to the recommended rate from Table 2, if necessary.
 - 9.1.5.5.2. Check and readjust the metering pump rate, if necessary.
- 9.1.5.6. Using the totalizer readings, pass a volume of water through the apparatus that equals the volume specified in Table 2.
- 9.1.5.7. Turn off the flow of water at the sample tap at the end of the sampling period and record the date, time of day, and totalizer reading onto a sample data sheet (see section 17.1). Although the totalizer reading may be affected by the addition of thiosulfate, the effect is insignificant and may be ignored.
- 9.1.5.8. Loosen the swivel female insert on the regulator module and disconnect the backflow regulator from the tap. Disconnect the cartridge housing module and the prefilter housing module, if used from the other modules.
- 9.1.5.9. Turn the filter housing(s) upside down and allow excess water to flow out. Turn the housing(s) upright and cover the quick connects on each end of the modules with sterile aluminum foil.

9.2. SHIPMENT OF SAMPLES

- 9.2.1. Pack the cartridge housing module(s) into an insulated shipping box.
- 9.2.2. Add 6-8 small ice packs (prefrozen at -20°C) or double bagged ice cubes around the cartridge housings to keep the sample cool in transit (the number of ice packs or bags may have to be adjusted based upon experience to ensure that the samples remain cold, but not frozen).
 - 9.2.2.1. Add an iButton (or other temperature recording device) to a location in the shipping box where it will not come in direct contact with the ice packs or bags.
 - 9.2.2.2. The temperature during shipment must be in the range of 1-10°C.
- 9.2.3. Drain and add the regulator and injector modules used.

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 9.2.4. Place the sample data sheet (protected with a closable plastic bag) in with the sample.
- 9.2.5. Drain and then cover the ends of the discharge module with foil. The discharge module may remain at the sampling site, if samples will be taken on a routine basis. If not, pack the module into the shipping box.
- 9.2.6. Close the insulated portion of the shipping box and tape to prevent any leakage of water. Close and label.
- 9.2.7. If the shipping box cannot be directly transported to the laboratory for virus analysis by close of business on the day collected or by the next morning, ship it to the laboratory by overnight courier.

9.3. LABORATORY HOLDING TIME AND TEMPERATURE

- 9.3.1. Record the date of arrival and the arrival condition on the sample data sheet packed with the sample. Print out the transit temperature reading from the iButton.
 - 9.3.1.1. Attach the readout of the iButton or other temperature-recording device for recording the temperature during shipment to the sample data sheet.
 - 9.3.1.2. Warning: The cartridge filters must arrive from the utility or other sampling site in a refrigerated, but not frozen, condition. The temperature during shipment must be in the range of 1-10°C.
 - 9.3.1.3. Brief transient temperatures outside the acceptable range associated with the initial packing and closing of the shipping box and its opening at the analytical laboratory may be ignored.
- 9.3.2. Filters must be refrigerated immediately upon arrival. Ideally, viruses should be eluted from filters within 24 h of the start of the sample collection, but all filters must be eluted within 72 h of the start of the sample collection.

10. FILTER ELUTION PROCEDURE

10.1. ELUTION EQUIPMENT SETUP

- 10.1.1. Attach sections of braided tubing to the inlet and outlet ports of the cartridge housing containing the cartridge filter (see Figure 4). Note: If a prefilter or more than one electropositive filter was used to collect a sample, each filter must be eluted and analyzed separately using the procedures below.
- 10.1.2. Place the sterile end of the tubing connected to the outlet of the cartridge housing into a sterile 2 L glass or polypropylene beaker.

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 10.1.3. Connect the free end of the tubing from the inlet port of the cartridge housing to the outlet port of a sterile pressure vessel and connect the inlet port of the pressure vessel to a positive air pressure source.

10.2. ELUTION

10.2.1. First elution

- 10.2.1.1. Elute NanoCeram or 1MDS filters with 500 mL or 1,000 mL of buffered 1.5% beef extract, pH 9.0 (item 7.2, prewarmed to room temperature), respectively, by opening the cartridge housing and adding a sufficient amount of beef extract to cover the filter. Close the housing and pour the remaining beef extract into the pressure container. An acceptable alternative to the use of a pressure vessel is to use a peristaltic pump and sterile tubing to push the remaining beef extract through the filter.
- 10.2.1.2. Replace the top of the pressure vessel and close its vent/relief valve.
- 10.2.1.3. Open the vent/relief valve (if present) on the cartridge housing and slowly apply sufficient pressure to fill the housing with beef extract. Close the vent/relief valve (if present) as soon as the buffered beef extract solution begins to flow from it. Carefully observe housings without vents to ensure that all trapped air has been purged.
- 10.2.1.4. Turn off the pressure and allow the solution to contact the filter for 1 minute. Wipe up spilled liquid with disinfectant-soaked sponge.
- 10.2.1.5. Increase the pressure to force the buffered beef extract solution through the filter(s). The solution should pass through the filter slowly to maximize the elution contact period. When air enters the line from the pressure vessel, elevate and invert the filter housing to permit complete evacuation of the solution from the filters. Note: Slow passage of the solution also minimizes foaming, which may inactivate some viruses; the addition of a few drops of sterile antifoam to minimize foaming is optional.
- 10.2.1.6. Turn off the pressure at the source and open the vent/relief valve on the pressure vessel.

10.2.2. Second elution

- 10.2.2.1. For the NanoCeram filter, repeat sections 10.2.1.1 to 10.2.1.6 using an additional 500 mL of buffered 1.5% beef extract, except increase the contact time in section 10.2.1.4 to 15 minutes. For a 1MDS filter, place the buffered beef extract

METHOD 1615. Enterovirus and Norovirus occurrence in water

from the 2 L beaker back into the cartridge housing and pressure vessel and repeat sections 10.2.1.2 to 10.2.1.6).

- 10.2.2.2. Turn off the pressure at the source and open the vent/relief valve on the pressure vessel. Combine the two 500 mL portions from the elution of NanoCeram filters. Record the total volume on the Virus Data Sheet (17.2). Thoroughly mix the eluate and proceed to the Organic Flocculation Concentration Procedure immediately.

11. ORGANIC FLOCCULATION CONCENTRATION PROCEDURE

11.1. ORGANIC FLOCCULATION

- 11.1.1. Place a sterile stir bar into the beaker containing the buffered beef extract eluate from the cartridge filter. Place the beaker onto a magnetic stirrer, and stir at a speed sufficient to develop a vortex. Minimize foaming (which may inactivate viruses) throughout the procedure by not stirring or mixing faster than necessary to develop a vortex.
- 11.1.2. Sterilize the electrode of a combination-type pH electrode as described in section 15.2.4. Calibrate the pH meter at pH 4 and 7.
- 11.1.3. Insert the sterile pH electrode into the beef extract eluate. Add 1.2 M HCl to the eluate dropwise while moving the tip of the pipette in a circular motion away from the vortex to facilitate mixing. Continue adding 1.2 M HCl until the pH reaches 3.5 ± 0.1 .
- 11.1.4. While continuing to monitor the pH, slowly stir the eluate for 30 minutes at room temperature. A precipitate will form during the 30 minutes stirring period. If pH falls below 3.4, add 1 M NaOH to bring it back to 3.5 ± 0.1 . Exposure to a pH below 3.4 may result in virus inactivation.
- 11.1.5. Remove the electrode from the beaker, and pour the contents of the beaker into a centrifuge bottle. To prevent the transfer of the stir bar into a centrifuge bottle, hold another stir bar or magnet against the bottom of the beaker while decanting the contents. The beef extract suspension will usually have to be divided into several centrifuge bottles.
- 11.1.6. Cap the bottle and centrifuge the precipitated beef extract suspension at $2,500 \times g$ for 15 minutes at 4°C .
- 11.1.7. Carefully pour off or aspirate the supernatant so as to not disturb the pellet, including any loose floc on top of the pellet, and then discard the supernatant.

11.2. RECONCENTRATED ELUATE

- 11.2.1. Place a stir bar into the centrifuge bottle that contains the precipitate.
- 11.2.2. Add 30 mL of 0.15 M sodium phosphate, pH 9.0. Note: A smaller volume (down to 15 mL) may be used, if the analytical laboratory's PE sample sets meet the performance requirements of Section 14.

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 11.2.3. Place the bottle onto a magnetic stirrer, and stir slowly until the precipitate has dissolved completely. Continue stirring for at least 10 minutes before proceeding to step 11.2.3.4. When the centrifugation is performed in more than one bottle, dissolve the precipitates in a total of 30 mL and combine into one bottle before proceeding to the next step. Significant virus loss can occur if the precipitates are not dissolved completely. Precipitates that prove to be difficult to dissolve can be treated using any of the following techniques:
 - 11.2.3.1. Break up the precipitate with a sterile spatula before or during the stirring procedure.
 - 11.2.3.2. Use a pipette repeatedly to draw the solution up and down during the stirring.
 - 11.2.3.3. Shake the precipitate at 160 rpm for 20 minutes on an orbital shaker in place of stirring.
 - 11.2.3.4. If the above procedures take longer than 20 minutes to dissolve the precipitate or if experience with the water matrix shows that precipitates are always difficult to manage, either slowly adjust the pH to 7.0 - 7.5 with 1.2 M HCl or resuspend the precipitate initially in 0.15 M sodium phosphate, pH 7.0 - 7.5. Use one of the above techniques to dissolve the precipitate and then slowly re-adjust the pH to 9.0 with 1 M NaOH. Mix for 10 minutes at room temperature before proceeding.
 - 11.2.3.5. Check the pH and readjust to 9.0 with 1 M NaOH, as necessary. Remove the stir bar.
 - 11.2.3.6. Centrifuge the dissolved precipitate at 4,000 x g for 10 minutes at 4°C. The centrifugation speed may be increased to 10,000 x g for 10 minutes at 4°C to facilitate the filtration step below. Record the centrifugation speed on the virus data sheet (section 17.2).
 - 11.2.3.7. Remove and collect the supernatant and discard the pellet. Adjust the pH of the supernatant to 7.0-7.5 slowly with 1.2 M HCl.
 - 11.2.3.8. Pretreat a sterilizing filter (item 6.3.7) or, for samples that are difficult to filter, a sterilizing filter stack (item 6.3.9) with 10-15 mL of 1.5% beef extract (item 7.2.4). Load the supernatant into a 50 mL syringe and force it through the filter. If the sterilizing filter or filter stack begins to clog badly, empty the loaded syringe into the bottle containing the unfiltered supernatant, fill the syringe with air, and inject air into filter to force any residual sample from it. Continue the filtration procedure with another filter.
- 11.2.4. Calculation of assay volumes and preparation of subsamples

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 11.2.4.1. Record the reconcentrated eluate volume (designated the **Final Concentrated Sample Volume** for cultural assays; **FCSV**) on the virus data sheet (section 17.2).
- 11.2.4.2. Calculate the **Assay Sample Volume (S)** for source and finished water samples using equation 1. **D (the Volume of Original Water Sample Assayed)** is the amount of reconcentrated eluate that must be assayed by Total Culturable Virus Assay (section 12) or processed for the Enterovirus and Norovirus Molecular Assay (section 12.2.11). This amount is 100 L for source water or 500 L for finished or ground waters. **TSV** is the **Total Sample Volume** from the **Virus Data Sheet**. The **Assay Sample Volume** is the volume of the reconcentrated eluate that represents 100 L of source water or 500 L of finished or ground waters. Record the S and D onto the Virus Data Sheet (section 17.2).

$$S = \frac{D}{TSV} \times FCSV \quad \text{Equation 1}$$

- 11.2.4.3. For example, if 1,800 L of a groundwater sample is passed through the NanoCeram filter and subsequently concentrated to 30 mL, then TSV equals 1,800 L, D equals 500 L, FCSV equals 30 mL, and $S = (500 \text{ L} / 1,800 \text{ L}) \times 30 \text{ mL} = 8.33 \text{ mL}$.
- 11.2.4.4. Prepare three subsamples of the reconcentrated eluate. Prepare subsamples 1 and 2 with a volume equal to 1.2 times the **Assay Sample Volume**. Prepare subsample 3 with the remaining volume. Label subsamples 1-3 with appropriate sampling information for identification.
- 11.2.4.4.1. Hold subsample 1 at 4°C for use with the Total Cultural Virus Assay if it can be assayed within 24 hours, otherwise freeze at -70°C.
- 11.2.4.4.2. Hold subsample 2 at 4°C and analyze using the Molecular Assay within 24 hours. Note: Freeze thawing leads to norovirus losses.
- 11.2.4.4.3. Freeze subsample 3 at -70°C for backup and archival purposes.
- 11.2.4.5. Determine the **Inoculum Volume** for the Total Culturable Virus Assay (section 12) by dividing the **Assay Sample Volume** by 10. Record the **Inoculum Volume** onto the Virus Data Sheet (section 17.2).
- 11.2.4.5.1. For ease of inoculation, a sufficient quantity of 0.15 M Na₂HPO₄, pH 7.0 - 7.5, may be added to the **Inoculum Volume** to give a more usable working **Final Inoculation Volume** (e.g., 1.0 mL). If this option is used, then record the **Final**

METHOD 1615. Enterovirus and Norovirus occurrence in water

Inoculation Volume onto the Virus Data Sheet (section 17.2) and substitute the term **Final Inoculation Volume** for each use of the **Inoculum Volume**, except where indicated.

11.2.4.5.2. For example, if an **Inoculum Volume** of 0.72 mL is to be placed onto each of 10 vessels, then for 10.5 vessels (with the extra 0.5 added to account for sample loss on the surface of the vessel used for the preparation), 7.56 mL of subsample will be needed (10.5×0.72 mL). The addition of 2.94 mL of 0.15 M Na_2HPO_4 , pH 7.0-7.5 ($10.5 \times (1 - 0.72)$ mL) to the 7.56 mL of subsample results in a mixture that contains the required **Inoculum Volume** per 1.0 mL.

11.2.4.6. Calculate the **Assay Sample Volume** for QC (section 8.4) samples by multiplying FCSV by 0.3. Calculate the **Inoculum Volume** by dividing the **Assay Sample Volume** by 10. Divide the **Final Concentrated Sample Volume** from QC samples into three subsamples and handle as described in step 11.2.4.4.

12. TOTAL CULTURABLE VIRUS ASSAY

12.1. QUANTAL ASSAY

12.1.1. Preparation of cell culture test vessels

12.1.1.1. Using 10 cell culture test vessels for every water sample to be tested, code each vessel with the water sample number, subsample number, analyst initials, and date using an indelible marker.

12.1.1.2. Return the cell culture test vessels to a $36.5 \pm 1^\circ\text{C}$ incubator and hold at that temperature until the cell monolayer is to be inoculated.

12.1.1.3. Decant and discard the medium from cell culture test vessels.

12.1.1.4. Wash the test vessels with a balanced salt solution (e.g., item 7.3.2.1) or maintenance medium without serum using a wash volume of at least 0.06 mL/cm^2 of surface area. Rock the wash medium over the surface of each monolayer several times and then decant and discard the wash medium. Do not disturb the cell monolayer during the wash procedure.

12.1.2. Inoculation of test samples

12.1.2.1. Rapidly thaw subsample 1, if frozen, and inoculate an amount equal to the **Inoculum Volume** or the **Final Inoculation Volume** (see step 11.2.4.5) onto each of 10 cell culture test vessels.

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 12.1.2.1.1. Note: Samples should be thawed in a 37°C water bath or under warm running water at about 37°C with shaking. Samples should be removed from the warm water as soon as the last ice crystal melt.
- 12.1.2.1.2. Note: The number of cell culture replicates was cut from the 20 required by the ICR standard method (19) to 10 to reduce labor costs. This reduction of replicates results in wider 95% confidence limits (c. 20-40%) and reduces the maximum virus titer that can be assayed without dilutions by about 25%. Laboratories assaying samples that are known to have more than about 20 MPN/100 L may assay subsample 1 using more than 10 replicates or by using dilutions as described in section 12.1.2.2.
- 12.1.2.1.3. Note: The analysis of a second subsample is not required for this method. Subsample 2 was used in the ICR method (19) to account for cytotoxicity. Samples with cytotoxicity should be assayed using dilutions as described in section 12.1.2.2.
- 12.1.2.1.4. **Warning!** Use at least a different pipetting tip or device for each set of samples to be inoculated.
- 12.1.2.2. For QC samples and for water samples known or suspected of having virus concentrations greater than 20 MPN/100 L, prepare five- and twenty five-fold dilutions of subsample 1 (or subsample 3, see section 12.1.2.1.3).
 - 12.1.2.2.1. Prepare a 1:5 dilution by adding a volume of subsample 1 equal to 0.1334 times the **Assay Sample Volume** (amount "a") to a volume of 0.15 M sodium phosphate (pH 7.0-7.5) equal to 0.5336 times the **Assay Sample Volume** (amount "b"). Mix thoroughly.
 - 12.1.2.2.2. Prepare a 1:25 dilution by adding amount "a" of the 1:5 diluted sample to amount "b" of 0.15 M sodium phosphate (pH 7.0-7.5).
 - 12.1.2.2.3. Inoculate 10 cell cultures each with undiluted subsample 1, subsample 1 diluted 1:5, and subsample 1 diluted 1:25, respectively, with an amount equal to the **Inoculum Volume**.
 - 12.1.2.2.4. Freeze the remaining portions of the 1:25 dilution at -70°C until the sample results are known. Thaw and perform additional 5-fold dilutions

METHOD 1615. Enterovirus and Norovirus occurrence in water

using the dilution format above if all replicates of the undiluted to 1:25-fold dilutions develop CPE.

12.1.2.3. Cell culture controls

12.1.2.3.1. **Negative Assay Control** – Inoculate three or more BGM cultures with a volume of sodium phosphate, pH 7.0 - 7.5, equal to the **Inoculum Volume** as a negative control. Run a **Negative Assay Control** with every group of subsamples inoculated onto cell cultures. If any **Negative Assay Control** develops CPE, all subsequent assays should be halted until the cause of the positive result is determined.

12.1.2.3.2. **Positive Assay Control** – Inoculate three or more BGM cultures with the **Positive Assay Control** (see item 7.3.3). Run a positive control with every group of subsamples inoculated onto cell cultures. This control will provide a measure for continued sensitivity of the cell cultures to virus infection. If any **Positive Assay Control** fails to develop CPE, all subsequent assays should be halted until the cause of the negative result is determined.

12.1.2.4. Record the date of inoculation on the **Virus Data Sheet** (section 17.2).

12.1.2.5. Rock the inoculated cell culture test vessels gently to achieve uniform distribution of inoculum over the surface of the cell monolayers. Place the cell culture test vessels on a mechanical rocking platform set at 1-5 oscillations per minute at room temperature.

12.1.2.6. Continue incubating the inoculated cell cultures for 80 - 120 minutes at room temperature to permit viruses to adsorb onto and infect cells. Note: If a rocking platform is not available, the vessels may be placed upon a level laboratory surface, but the vessels should be rocked every 15-20 minutes during the adsorption period to prevent cell death in the middle of the vessels from dehydration.

12.1.2.7. Add liquid maintenance medium (see Item 3.7) and incubate at $36.5 \pm 1^{\circ}\text{C}$.

12.1.2.7.1. Warm the maintenance medium to $36.5 \pm 1^{\circ}\text{C}$ before placing it onto cell monolayers.

12.1.2.7.2. Add the medium to the side of the cell culture vessel opposite the cell monolayer.

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 12.1.2.7.3. **Warning!** Never touch the pipetting device to the inside rim of the cell culture test vessels during medium addition. This step represents the most likely place where cross contamination of cultures can occur. Cross contamination will result in invalid MPN values and can cause false positive results. Laboratories must ensure that analysts take great precaution in performing this step.
- 12.1.2.7.4. If CPE has not started to develop, the cultures may be re-fed with fresh maintenance medium after 4-7 days.
- 12.1.3. CPE development
 - 12.1.3.1. Examine each culture microscopically for the appearance of CPE daily for the first three days and then every couple of days for a total of 14 days.
 - 12.1.3.2. Freeze cultures at -70°C when more than 75% of the monolayer has developed CPE. Freeze all remaining cultures, including controls, after 14 days.
- 12.1.4. Perform a second passage for confirmation. Confirmation passages may be performed in small vessels or multiwell trays, however, it may be necessary to distribute the inoculum into several vessels or wells to insure that the inoculum volume is less than or equal to 0.04 mL/cm² of surface area.
 - 12.1.4.1. Thaw all the cultures, including the negative and positive assay controls, to confirm the results of the previous passage. Refreeze at least 2 mL of the medium from each vessel at -70°C for optional analysis by molecular methods (see step 13.3.2).
 - 12.1.4.2. Filter at least 10% of the medium from each vessel that was positive for CPE through separate 0.22 µm sterilizing filters (item 6.3.8). If the medium is difficult to filter, it can be centrifuged at 1,500 to 18,000 x g prior to filtration.
 - 12.1.4.3. Prepare fresh cell culture test vessels as described in step 12.1.1. Inoculate the fresh cultures with the thawed medium from all negative samples (step 12.1.4.1) and the filtered medium from step 12.1.4.2 using an inoculation volume that represents 10% of the medium from the first passage.
 - 12.1.4.4. Repeat sections 12.1.2.4 to 12.1.3.1.
- 12.1.5. Score cultures that developed CPE in both the first and second passages as confirmed positives.
 - 12.1.5.1. Perform a third passage as described in Step 12.1.3.2 through 12.1.4.4 on any cell culture vessels that were negative during

METHOD 1615. Enterovirus and Norovirus occurrence in water

the first passage and positive in the second passage and with the negative assay controls. Other vessels that were either negative or positive in both the first and second passages do not need to be carried through the third passage.

- 12.1.5.2. Score cultures that develop CPE in both the second and third passages as confirmed positives.

12.2. VIRUS QUANTITATION

- 12.2.1. Record the total number of confirmed and not confirmed positive and negative cultures for each subsample onto a **Total Culturable Virus Data Sheet** (section 17.3).
- 12.2.2. Transfer the number of cultures inoculated and the number of confirmed positive cultures from the **Total Culturable Virus Data Sheet** for each sample to the **Quantitation of Total Culturable Virus Data Sheet** (section 17.4).
- 12.2.3. Calculate the MPN/mL value (M_{mL}) and the upper (CL_{uml}) and lower (CL_{lmL}) 95% confidence limits/mL using the number of confirmed positive cultures from step 12.2.2 and the MPN computer program supplied by U.S. EPA (www.epa.gov/microbes). If dilutions are required, calculate the MPN/mL value and 95% confidence limits using the confirmed values from all dilutions, including dilutions with all positive or negative samples.
 - 12.2.3.1. Change the MPN program default settings as shown in Table 3.
 - 12.2.3.2. Note that the **Inoculum Volume** rather than the **Inoculation Volume** (see step 11.2.4.5) must be used.
 - 12.2.3.3. The MPN program will run on Windows XP and later versions. It has been re-designed for calculation of both standard bacterial and viral MPN values. All entries are saved in a default database and can be viewed to check for data entry errors using the View History selection under the Tools menu. Each program run can also be saved into Word, Excel, or text files for transfer to lab notebooks or to Laboratory Information Management Systems.
- 12.2.4. Place the values obtained onto the **Quantitation of Total Culturable Virus Data Sheet** (section 17.4).
- 12.2.5. Calculate the MPN per L value (M_L) of the original water sample using equation 2 where **S** equals the **Assay Sample Volume** and **D** equals the **Volume of Original Water Sample Assayed** (the values for **S** and **D** can be found on the **Virus Data Sheet**). Record the value of M_L onto the **Virus Data Sheet** (section 17.2). For M_{mL} values of 0, calculate the sample detection limit rather than the M_L value by dividing 1 by **D**. Report as equal to or less than the calculated detection limit. For samples where more than one cartridge filter or a prefilter was used, place the total

METHOD 1615. Enterovirus and Norovirus occurrence in water

MPN/L and Confidence Limits/L (from steps 12.2.7 and 12.2.9) values for all filters on the **Virus Data Sheet** and place the individual totals for each filter under “Other Comments.”

$$M_L = \frac{M_{mL} S}{D} \quad \text{Equation 2}$$

- 12.2.6. For example, if the sample described in the example in step 11.2.4.2, which has an **Inoculum Volume** equal to 0.833 mL, had 4 positive replicates, the MPN/mL value is 0.61 with 95% Confidence Limits of 0.12 to 1.31. The MPN per L value then equals (0.61 MPN/mL x 8.33 mL)/500 L = 0.0102.

- 12.2.7. Calculate the lower 95% confidence limit per L value (CL_L) for each water sample using equation 3 where CL_{mL} is the lower 95% confidence limit per milliliter from the Quantitation of Total Culturable Virus Data Sheet. Record the limit per L values on the Virus Data Sheet.

$$CL_L = \frac{CL_{mL} S}{D} \quad \text{Equation 3}$$

- 12.2.8. Continuing with the example from step 12.2.5, $CL_L = (0.12 \text{ MPN/mL} \times 8.33 \text{ mL})/500 \text{ L} = 0.002$.

- 12.2.9. Calculate the upper 95% confidence limit per L value (CL_U) using equation 4 where CL_{umL} is the upper 95% confidence limit per milliliter from the **Quantitation of Total Culturable Virus Data Sheet**. Record the limit per L values on the **Virus Data Sheet**.

$$CL_U = \frac{CL_{umL} S}{D} \quad \text{Equation 4}$$

- 12.2.10. Continuing with the example from step 12.2.5, $CL_U = (1.31 \text{ MPN/mL} \times 8.33 \text{ mL})/500 \text{ L} = 0.0218$.

- 12.2.11. Calculate the total MPN value and the total 95% confidence limit values for each QC samples by multiplying the values per milliliter by S and dividing by 0.3.

13. ENTEROVIRUS AND NOROVIRUS MOLECULAR ASSAY

The molecular assay uses RT-qPCR based upon standard curves to provide a quantitative estimate of enterovirus and norovirus genomic copies per liter in environmental and drinking waters. Only microliter volumes can be analyzed by RT-qPCR, so the procedure includes additional concentration of any viruses present in the sample beyond that required for culture. Surface and ground waters may contain substances that interfere with RT-qPCR, so the assay uses an RNA extraction kit to reduce inhibition and a control to identify samples that are inhibitory to RT-qPCR. The assay uses primers and probes from the scientific literature (Table 4). The primer/probe sets are designed to detect many enteroviruses and noroviruses. Standard curves are prepared from Armored RNA[®] reagents that contain the target sequence for the primer/probe sets. Armored RNA was chosen for standard curves because it is difficult to obtain high titered norovirus stocks.

METHOD 1615. Enterovirus and Norovirus occurrence in water

13.1. PRELIMINARY PROCEDURES

- 13.1.1. Purchase the primers and probes shown in Table 4.
- 13.1.2. Prepare 100 μ M stock solutions of each oligonucleotide primer (probes should come from the manufacturer at 100 μ M solutions).
 - 13.1.2.1. Centrifuge the vials containing primers in a microcentrifuge for 30 seconds.
 - 13.1.2.2. Dissolve the primers in a volume of molecular or PCR grade water (e.g., Roche Cat. No. 03 315 932 001) that equals the number of nanomoles of primer shipped (as identified on the specification sheet from the manufacturer) times 10 (in μ l; e.g., if a primer contains 51.0 nmol; resuspend in 510 μ l).
 - 13.1.2.3. Measure the absorbance at 260 nm. Calculate the total absorbance by multiplying the absorbance value by the volume used to resuspend the primer (and by any dilution required to obtain the absorbance reading). Confirm that the reading is the same as the total absorbance value recorded on the manufacturer's specification sheet. Note: Probes should be quality checked using this same procedure.
 - 13.1.2.4. Aliquot the stock solutions into small quantities to avoid multiple freeze thaw cycles and store at -20°C.
- 13.1.3. Prepare 10 μ M primer and probe working solutions by diluting the stock solutions 1:10 (or by a dilution that compensates for any differences between the absorbance reading obtained in Section 13.1.2.3 and the manufacturer's specification sheet) in molecular or PCR grade water. Aliquot stocks and store at -20°C.
- 13.1.4. Note: Preparation of primers and probes must be performed in a clean room or other location to minimize the possibility of false positive reactions. A clean room or location is one in which molecular and microbiological procedures are not performed.

13.2. TERTIARY CONCENTRATION OF SUBSAMPLE 2

- 13.2.1. For each sample to be analyzed, label a Vivaspin 20 unit with the sample number, analyst's initials and date, and fill the unit with PBS, 0.2% BSA (item 7.4.5). Soak at least 2 hours at room temperature or overnight at 4°C.
- 13.2.2. Discard the PBS, 0.2% BSA from the Vivaspin 20 unit and add an amount of the appropriate subsample 2 (see section 11.2.4.4.2) equal to the **Assay Sample Volume (S)**.
- 13.2.3. Centrifuge at 3,000 x g with swinging buckets until the sample has been concentrated down to about 50 μ L.
- 13.2.4. Add 1 mL of sterile 0.15 M sodium phosphate, pH 7-7.5, and repeat step 13.2.3. Repeat one additional time.

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 13.2.5. Transfer the concentrate to a 1.5 ml microcentrifuge tube. Measure the volume and add 0.15 M sodium phosphate, pH 7-7.5 to bring the total volume to 0.4 mL.
- 13.2.6. Immediately proceed to step 13.3 or hold at 4°C for no more than 72 hours. Note: Freeze/thawing leads to norovirus losses.

13.3. NUCLEIC ACID ISOLATION

- 13.3.1. Preliminary procedures
 - 13.3.1.1. Add 310 µL of Buffer AVE (item 7.4.8) to a vial of carrier RNA (from item 7.4.7) to give a final concentration of 1 µg/µL.
 - 13.3.1.2. Aliquot and store at -20°C. Prepare a sufficient number of aliquots so that each aliquot does not have to be frozen and thawed more than three times.
 - 13.3.1.3. Add carrier RNA to Buffer AVL (item 7.4.7; note: do not use the Buffer AL that comes with the Qiagen kit) to give a concentration of 0.027 µg/µL by adding, per sample, 5.6 µL of carrier RNA (from step 13.3.1.2) to 200 µL of Buffer AVL (e.g., 5.6 µL carrier RNA × number of samples + 200 µL Buffer AVL × number of samples).
- 13.3.2. For each sample to be processed, label a 1.5 mL microcentrifuge tube and add 200 µL of the appropriate sample from section 13.2.5 (or from section 12.1.4.1 for confirmation of culture positive samples) and vortex briefly to mix. Freeze any remaining tertiary concentrate at -70°C.
- 13.3.3. Add 200 µL of Buffer AVL with carrier RNA from step 13.3.1.3. Vortex for 15 seconds.
- 13.3.4. Incubate at 56°C for 10 minutes.
- 13.3.5. Centrifuge at >5,000 rpm for 5 seconds in a microcentrifuge.
- 13.3.6. Add 200 µL of ethanol. Vortex for 15 seconds and then centrifuge for about 5 seconds.
- 13.3.7. Add the mixture to a QIAamp Mini Spin column, but avoid wetting the rim of the tube. Close the cap.
- 13.3.8. Centrifuge at 6,000 x g for 1 minute. Check to determine if the sample has completely passed through the column. If it has not, centrifuge again for 1 minute at 10,000-20,000 x g or for longer times until the sample has completely passed through the column.
- 13.3.9. Place the Mini Spin column into a clean 2 mL collection tube (item 7.4.9) and discard the collection tube containing the filtrate.
- 13.3.10. Add 500 µL of Buffer AW1 without touching the tube rim.
- 13.3.11. Centrifuge at 6,000 x g for 1 minute. Again, transfer the column to a clean collection tube and discard the tube containing the filtrate.

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 13.3.12. Add 500 μL of Buffer AW2 without touching the tube rim.
- 13.3.13. Centrifuge at 20,000 $\times g$ for 3 minutes. Again, transfer the column to a clean collection tube and discard the tube containing the filtrate.
- 13.3.14. Centrifuge at 20,000 $\times g$ for 1 minute.
- 13.3.15. Add 40 units of RNasin to a clean microcentrifuge tube. Transfer the column from the collection tube to the 1.5 mL microcentrifuge tube and discard the collection tube. Alternatively, RNasin can be added to an amount of Buffer AE sufficient for the number of samples to be eluted at a concentration of 400 units/mL in place of adding it to the microcentrifuge tubes.
- 13.3.16. Add 50 μL of Buffer AE to the column. Incubate at room temperature for 1 minute and then centrifuge for 1 minute at 6,000 $\times g$.
- 13.3.17. Repeat step 13.3.16. Remove and discard the column.
- 13.3.18. Proceed immediately to step 13.4 or prepare aliquots and store the RNA at -70°C until it can be assayed.

13.4. REVERSE TRANSCRIPTION (RT)

- 13.4.1. Preliminary procedures (to be performed in a clean room)
 - 13.4.1.1. Label PCR plates or tubes with appropriate sample numbers.
 - 13.4.1.2. Prepare RT master mix 1 and 2 using the guide in Table 5. The amounts shown for the volume per master mix can be scaled up or down according to the number of samples that need to be analyzed.
 - 13.4.1.3. Vortex the master mixes after the addition of all ingredients.
 - 13.4.1.4. Centrifuge at $>5,000$ rpm for 10 seconds in a microcentrifuge.
- 13.4.2. Aliquot 16 μL of RT master mix 1 to the labeled PCR tubes or plate wells.
 - 13.4.2.1. Run every environmental water, QC, and PE sample in triplicate by adding 6.7 μL of the appropriate sample to each of the tubes or plate wells labeled for that sample. Note: It is not necessary to prepare 1:5 and 1:25 dilutions of the QC samples as done for the culture assay (section 12.1.2.2). See Figure 4 for a schematic of the RT-qPCR process.
 - 13.4.2.2. Add 6.7 μL of molecular grade water to one or more tubes or plate wells as no template controls (NTC). Include at least one NTC for the replicates associated with every fourth water sample run on a plate; NTC controls must be distributed throughout the plate. Note: If any NTC control is positive, the cause of the false positive value should be investigated. After fixing the cause of the problem, all samples on the plate must be rerun.

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 13.4.2.3. Close the tubes or seal the plates and heat at 99°C for 4 minutes, followed by quenching on ice or a hold temperature of 4°C.
- 13.4.3. Add 17.3 µL of RT mix 2 to each tube or well.
- 13.4.4. Centrifuge at >5,000 rpm for 10 seconds in a microcentrifuge at 4°C.
- 13.4.5. Run at 25°C for 15 minutes, 42°C for 60 minutes, 99°C for 5 minutes, and then hold at 4°C for up to 2 hours until PCR is performed. Note: Thermal cyclers from a number of different manufacturers can be used for this and the following real-time quantitative PCR step. Analysts must follow the manufacturers' instructions for set-up, runs, and analysis for the instrument used.
- 13.4.6. Centrifuge at >5,000 rpm for 10 seconds in a microcentrifuge.
- 13.4.7. Proceed immediately to step 13.5 or store reverse transcribed samples at -70°C until they can be processed.

13.5. REAL-TIME QUANTITATIVE PCR (qPCR)

- 13.5.1. Preliminary procedures
 - 13.5.1.1. Label PCR plates or tubes with appropriate sample numbers.
 - 13.5.1.1.1. Note: each sample will require 15 plate wells or tubes (i.e., 3 RT replicates times 5 PCR assays; see Figure 4).
 - 13.5.1.1.2. To save reagents, the HGV inhibition control assay may be run before all other qPCR assays (see section 13.5.8).
 - 13.5.1.2. Prepare PCR master mixes using the guides in Tables 6-10. The amounts shown for the volume per master mix can be scaled up or down according to the number of samples that need to be analyzed.
 - 13.5.1.3. Vortex the master mix after the addition of all ingredients.
 - 13.5.1.4. Centrifuge at >5,000 rpm for 10 seconds in a microcentrifuge.
 - 13.5.1.5. Dispense 14 µL of the appropriate mix to the labeled plates or tubes.
- 13.5.2. Add 6 µL of the appropriate sample to each tube (see Figure 4).
- 13.5.3. Run on a thermal cycler with a setting of 1 cycle at 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute.
- 13.5.4. Analyze the results of each run to calculate the **Genome Copy (GC)** numbers of unknown samples according to the instructions of the manufacturer of the thermal cycler used based upon the standard curve samples described in step 13.5.9.

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 13.5.5. Record the **GC** values on the **Molecular Virus Results Data Sheet** (section 17.6) of each replicate and mean and standard deviation of the three replicates for each sample. Include non-detects (zeros) in the calculation of the mean
- 13.5.6. Calculate the **Genomic Copies per L (GC_L)** for each sample using equation 5 and the mean **GC** value from step 13.5.4, where **DF** equals the reciprocal of any dilution performed to compensate for inhibition (see step 13.5.8; e.g., 5 and 25, or 1 for undiluted samples) and **D** equals the **Volume of Original Water Sample Assayed** (see step 11.2.4.2).

$$GC_L = \frac{GC \times 199 \times DF}{D} \quad \text{Equation 5}$$

For example, if the PCR assay from the sample example described in step 11.2.4.2 detects 15 genomic copies in a 1:5 dilution, then the number of genomic copies per 100 L is $100 \times ((15 \times 199 \times 5)/500 \text{ L}) = 2985$. Note: 199 is the total dilution factor for the volume reductions that occur in steps 13.2 through 13.5.

- 13.5.6.1. Record the **GC_L** value on the **Molecular Virus Results Data Sheet** (section 17.6).
- 13.5.6.2. For water samples with a mean value of zero, report the Genomic Copies per L as less than or equal to the detection limit (i.e., $\leq 1/D$).
- 13.5.7. Calculate the genomic copies of QC samples by multiplying the mean genomic copy value by 199 and dividing by 0.3.
- 13.5.8. Inhibition Control
- 13.5.8.1. Hepatitis G Armored RNA is used as an inhibition control rather than specific enterovirus and norovirus to reduce the possibility of cross-contamination that could occur between seeded and unseeded samples with the actual virus groups being tested.
- 13.5.8.2. Before running any water samples, run ten replicate RT and hepatitis G qPCR assays to determine the mean hepatitis G Cq value and standard deviation.
- 13.5.8.2.1. Process a volume of FCSV from at least two negative QC controls (step 11.2.4.6) equal to the **Assay Sample Volume (S)** using steps 13.2 to 13.3. Use 6.7 µL of the RNA from one of these samples; hereafter designated “negative FCSV” for five of the replicates and 6.7 µL from the other sample for the other five replicates.
- 13.5.8.2.2. Calculate the mean quantitation cycle (Cq) value and standard deviations. Record the results on the **Molecular Virus Results Data Sheet** (section

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 17.6). The mean value should be between 25 and 32 Cq units. The standard deviation of the mean should be <0.3 units.
- 13.5.8.2.3. Note: If the mean value is not between 25 and 32 Cq units, readjust the amount of Hepatitis G Armored RNA added to RT Master Mix 1 (see Table 5) and repeat step 13.5.8.2 until the value is within the acceptable range.
- 13.5.8.3. Compare the hepatitis G Cq values obtained with all samples against the value calculated in step 13.5.8.2. If the value in the unknown samples is more than 1 Cq value higher than that calculated in step 13.5.8.2 dilute the unknown sample 1:5 and 1:25 in molecular grade (nuclease-free) water.
- 13.5.8.4. Re-run the sample along with the 1:5 and 1:25 dilution.
- 13.5.8.5. Because some samples will demonstrate some level of inhibition, the hepatitis G assay can be run with all samples prior to doing the other qPCR assays as stated in step 13.5.1.1.2. All other assays can then be run without dilution for samples giving no inhibition and with the appropriate dilution for all other samples.
- 13.5.8.6. Calculate the sample concentration using the highest dilution for which the hepatitis G Cq values are within 1 unit of the value calculated in step 13.5.8.2. If the inhibition control fails again and the sample Cq value is lower than 38, re-run the sample at higher five-fold dilutions. If any sample run at the higher dilution fail the inhibition control again or if any unknown samples are below the detection limit (e.g., Cq values of 45 or higher), list the sample as a potential false negative sample on the **Molecular Virus Results Data Sheet** (section 17.6)
- 13.5.9. Preparation of standard curves. Note: Analysts must run new standard curves (a single standard curve for each Armored RNA standard) with every set of unknown samples run together or use calibrators (see step 13.5.10) with stored standard curves, if the thermal cycler allows standard curves to be stored.
- 13.5.9.1. Dilute enterovirus, norovirus GI and norovirus GII Armored RNA in negative FCSV to give a concentration of 2.5×10^6 particles/250 μ L (i.e., 1×10^7 /mL) based upon the concentration of the Armored RNA lots supplied. Note: unless specified otherwise by EPA, transcribed RNA from plasmids containing the appropriate viral sequence or poliovirus, norovirus GI, and norovirus GII stocks may be substituted for the Armored RNA.

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 13.5.9.1.1. Transcribed RNA must be titrated using standard methods, e.g., see reference (32), and genomic copies per mL substituted for particles per mL above.
- 13.5.9.1.2. Virus stocks must be titrated using RT-qPCR on endpoint serial dilutions and ten replicates per dilution for each stock. The MPN calculator must then be used to obtain the titer of the stock and the MPN value substituted for particles per mL above. If the efficiency of the standard curve is in the acceptable range (see step 13.5.9.7), genome copies per mL can be substituted for MPN/mL.
- 13.5.9.2. Prepare 6 ten-fold dilutions to give concentrations of 1×10^6 , 1×10^5 , 1×10^4 , 1×10^3 , 1×10^2 , 1×10^1 and 1×10^0 particles/mL. Prepare by serially adding 25 μ L of Armored RNA to 225 μ L of negative FCSV to create each dilution with vortexing for 5-15 seconds before proceeding to the next higher dilution.
- 13.5.9.3. Run 200 μ L of each dilution separately through steps 13.3-13.5.4 using the volumes described in the steps and only the specific primers/probe for the Armored RNA standard. This will result in six standards with 10^0 to 10^5 particles per assay.
- 13.5.9.4. Calculate the standard curve slope and R^2 values for each standard curve by plotting Cq values against the log of the concentration for each point or, if available, by using the slope and R^2 values determined by the qPCR instrument.
- 13.5.9.5. Calculate the mean of the slopes and standard deviation of the mean for the three runs and the mean of the R^2 values. Note: An acceptable standard curve will have a mean R^2 value >0.97 and a standard deviation of <0.25 . Standard deviations of 0.25 or higher represent errors in preparing dilutions or in pipetting.
- 13.5.9.6. Calculate the percent amplification efficiency using equation 6. Record the results on the **Molecular Virus Protocol Data Sheet** (section 17.5).
$$\%Efficiency = 100 \times (10^{-1/slope} - 1) \quad \text{Equation 6}$$
- 13.5.9.7. For example, the ideal efficiency occurs when the slope equals -3.32, where the % Efficiency = $100 \times (10^{-1/-3.33} - 1) = 100 \times (2.0 - 1) = 100$. Note: An acceptable standard curve will have an amplification efficiency of 80-110%.
- 13.5.9.8. Standard curves that meet the criteria specified in steps 13.5.9.5 and 13.5.9.6 above must be used to calculate genomic copies of unknown samples in step 13.5.4.
- 13.5.10. Preparation of calibrators and standard curves for use with calibrators

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 13.5.10.1. Stored standard curves must be used with calibrators. The stored standard curves must be based upon the mean of three runs with each set of standards. New standard curve sets must be generated as described above every eighth sample batch (see section 3) or every two months, whichever comes first, or anytime a calibrator fails twice in a row to meet acceptance criteria.
- 13.5.10.2. To prepare calibrators for each virus standard, choose the dilution from the standard curve that gives the Cq value closest to, but not greater than 32. Note: calibrators must be run with all unknown samples if stored standard curves are used, but may be run even if standard curves are run with each test as an additional quality check.
- 13.5.10.3. Prepare the dilution corresponding to the chosen value in negative FCSV and extract the RNA as described in step 13.3. Prepare a sufficient number of dilutions to last for the entire study, taking into consideration that each 200 µL extraction will yield sufficient material for about 20 runs. Aliquot into single run batches and store at -70°C.
- 13.5.10.4. Run a set of at least ten calibrators from each Armored RNA standard. Calculate the mean Cq value and standard deviations. Record the mean and standard deviation values as the **Target Value** on the **Molecular Virus Results Data Sheet** (section 17.6). The standard deviation must be less than 0.25 units.
- 13.5.10.5. Run all calibrators with every set of unknown samples. Accept a run if the value of the calibrator for each corresponding PCR assay falls within 1.0 Cq units of the calibrators' mean values. Record the observed value for each target virus as the **Actual Value** on the **Molecular Virus Results Data Sheet** (section 17.6).
- 13.5.10.6. Reject sample sets run in a PCR assay where the calibrator for that assay falls outside the acceptance criteria. Repeat the run once upon failure. If the assay fails again, generate a new standard curve or take steps to determine the cause of the failure.

14. METHOD PERFORMANCE

14.1. CULTURABLE ASSAY

- 14.1.1. This method is subject to a number of biases that reduce its precision and accuracy. The isoelectric point of the virus particle affects its ability to bind to, and be eluted from, electropositive filters. The isoelectric point can vary significantly across virus species and even within members of the same species. Other capsid and matrix related characteristics and

METHOD 1615. Enterovirus and Norovirus occurrence in water

substances could affect virus recovery at various stages of the method. The passage number of the BGM cell line and the media used to passage and maintain cells is known to affect the ability of viruses to replicate in cells. The performance characteristics given below are based upon Sabin poliovirus type 3 and may not be reflective of other viruses that are detected by this method. The best performance data for the method comes from the performance evaluation (PE) samples that were analyzed during the ICR as described (19). In total, 12 laboratories with 25 ICR-approved analysts analyzed 828 PE samples, consisting of low (<300 MPN per filter), medium (300-1,500 MPN per filter) and high (>1,500 MPN per filter) virus levels. The mean interlaboratory recovery was 56% with a coefficient of variation (CV) of 92%, a false negative rate of 1.3%, and a false positive rate of 1.1%. The highest mean recoveries values (71%) were obtained with the PE samples containing the low virus levels. Table 11 gives the mean recovery and CV value ranges for individual analysts and intralaboratory variation. Although Method 1615 uses a different electropositive filter than the ICR study, both filters have been shown to give the same recoveries in a single (24) and a four laboratory validation study (unpublished data).

- 14.1.2. The detection limit of the culture method is 0.01 MPN/L for surface water and 0.002 MPN/L for groundwater.
- 14.1.3. Acceptance limits of PE and QC samples is set for this method at a mean recovery of 35 to 150% with a CV of less than or equal to 70% based upon the 90% confidence limits of the data from the ICR PE analyses.

14.2. MOLECULAR PROCEDURE

- 14.2.1. The molecular procedure is subject to the same bias as the culturable procedure in terms of virus adsorption and recovery from the electropositive filters and secondary concentration procedures. Additional bias can occur during tertiary concentration, RNA elution and RT-qPCR. The method was tested using seven groundwater samples from five different wells with a range of physicochemical characteristics. In addition to bias from matrix effects, these tests may have had additional bias because they were performed as described in section 8.6. The seven groundwater samples gave a mean recovery of 26% with a recovery range of 5-60% and a CV of 73%. These same samples were also tested for norovirus recovery using Murine norovirus and Murine norovirus specific primers and probe with the Method 1615 protocols. Mean recovery of Murine norovirus was 35% with a recovery range of 7-63% and a CV of 69%.
- 14.2.2. As with the culture method, the acceptance criteria for the molecular procedure is based upon the variation among PE samples seeded with Sabin poliovirus type 3. The acceptance criteria for the molecular procedure is a mean recovery of 20 to 150% with a CV of less than or equal to 75% based upon genomic copies detected by the molecular

METHOD 1615. Enterovirus and Norovirus occurrence in water

procedure and the genomic copies seeded onto the PE samples, unless specified otherwise by EPA for specific applications.

- 14.2.3. The detection limit of the molecular method based upon the overall detection limit of the RT-qPCR assay and the volume of water sample assayed. The detection limit for the poliovirus assay is about 2 and 0.4 genomic copies/L for surface water and groundwater, respectively. The detection limit can be increased by running more than three RT-qPCR replicates from each sample.

14.3. PERFORMANCE RECORD

The laboratory should maintain a record of the performance of QC and PE samples for both the cultural and molecular portions of this method and measure each individual performance against the ongoing laboratory values and the performance data given in this section. This should be done by continuously updating the mean recovery and coefficient of variation values with the addition of each new QC and PE sample.

15. STERILIZATION AND DISINFECTION

15.1. GENERAL GUIDELINES

- 15.1.1. Use aseptic techniques for handling test waters, eluates and cell cultures.
- 15.1.2. Sterilize apparatus and containers that will be exposed to test waters and all solutions that will be added to test waters unless otherwise indicated. Thoroughly clean all items before final sterilization using laboratory standard operating procedures.
- 15.1.3. Sterilize all contaminated materials before discarding.
- 15.1.4. Disinfect all spills and splatters.

15.2. STERILIZATION TECHNIQUES

- 15.2.1. Solutions
 - 15.2.1.1. Sterilize all solutions, except those used for cleansing, standard buffers, HCl, NaOH, and disinfectants by autoclaving them at 121°C for at least 15 minutes.
 - 15.2.1.2. HCl, NaOH, and disinfectants used are self-sterilizing. When autoclaving buffered beef extract, use a vessel large enough to accommodate foaming.
- 15.2.2. Autoclavable glassware, plasticware, and equipment
 - 15.2.2.1. General considerations: Add sufficient dH₂O to all vessels to be autoclaved to equal about 1-2% of the vessel's rated volume. Water speeds the sterilization process by enhancing the transfer of heat. Place large vessels on their sides in the autoclave, if possible, to facilitate the displacement of air in the vessels by flowing steam.

METHOD 1615. Enterovirus and Norovirus occurrence in water

- 15.2.2.2. Cover the openings into autoclavable glassware, plasticware, and equipment loosely with aluminum foil before autoclaving. Autoclave at 121°C for at least 30 minutes.
- 15.2.2.3. Glassware may also be sterilized in a dry heat oven at a temperature of 170°C for at least 1 hour.
- 15.2.2.4. Sterilize stainless steel vessels (dispensing pressure vessel) in an autoclave at 121°C for at least 30 minutes. Vent-relief valves on vessels so equipped must be open during autoclaving and closed immediately when vessels are removed from autoclave.
- 15.2.2.5. Pre-sterilize 1MDS filters and prefilters by wrapping the filters in Kraft paper and autoclaving at 121°C for 30 minutes. Warning: Do not autoclave the NanoCeram filters specified in item 6.1.2.4. These filters are sterilized by the manufacturer and have housings that cannot be autoclaved.
- 15.2.3. Sterilize instruments, such as scissors and forceps, by immersing them in 95% ethanol and flaming them between uses.
- 15.2.4. Sodium hypochlorite sterilization - Sterilize pumps, plasticware (filter housings) and tubing that cannot withstand autoclaving, and vessels that are too large for the autoclave with sodium hypochlorite (item 7.1.4). Ten-inch cartridge prefilters, but not NanoCeram or 1MDS filters, may be presterilized with sodium hypochlorite as an alternative to autoclaving. Filter apparatus modules should be disinfected after use by sterilization and then cleaned according to laboratory standard operating procedures before final sterilization.
 - 15.2.4.1. Sterilize the items that cannot be autoclaved by recirculating or immersing the items in 0.525% sodium hypochlorite for 30 minutes. pH electrodes should be sterilized with 0.525% hypochlorite for at least 5 minutes.
 - 15.2.4.2. Drain the hypochlorite from the objects being sterilized and rinse in sterile water.
 - 15.2.4.3. Dechlorinate by recirculating or immersing the items in a solution containing 50 mL of 1M sodium thiosulfate per liter of sterile dH₂O. . Ensure that the sodium hypochlorite (step 15.2.4.1) and sodium thiosulfate (step 15.2.4.3) solutions come in full contact with all surfaces when performing this procedure.
 - 15.2.4.4. Cover the apparatus module ends and the injector port(s) with sterile aluminum foil.
 - 15.2.4.5. Place the injector module and tubing into a sterile bag or wrapping in such a way that they may be removed without contaminating them.

METHOD 1615. Enterovirus and Norovirus occurrence in water

15.2.5. Contaminated materials

- 15.2.5.1. Autoclave contaminated materials for at least 30 minutes at 121°C. Be sure that steam can enter contaminated materials freely.
- 15.2.5.2. Many commercial disinfectants do not adequately kill enteric viruses. To ensure thorough disinfection, disinfect spills and other contamination on surfaces with either a solution of 0.5% iodine (item 7.2.2) or 0.525% sodium hypochlorite. The iodine solution has the advantage of drying more rapidly on surfaces than sodium hypochlorite, but may stain some surfaces.

METHOD 1615. Enterovirus and Norovirus occurrence in water

16. TABLES AND FIGURES

Table 1. Viruses Detected by EPA Method 1615

Virus Genus or Species	Detected by TCVA ^a	Detected by qPCR
<i>Human enterovirus A</i>	Some serotypes	Yes
<i>Human enterovirus B</i>	Most serotypes	Yes
<i>Human enterovirus C</i>	Some serotypes	Yes
<i>Human enterovirus D</i>	Some serotypes	Yes
<i>Norovirus</i> genogroup I and II	No	Many genotypes
<i>Mammalian orthoreovirus</i>	Yes	No

^a TCVA – Total Cultural Virus Assay (see section 12)

Table 2. Specified and Recommended Sample Volumes

Water Type	Flow Rate ^a (L/min)	Sampling duration (hours)	Sample Volume (liters) ^{b,c}
Sewage effluent	10	0.2	120 ^d
Surface	10	0.6	360 ^e
Finished/Groundwater	10	3.0	1,800 ^f
Finished/Groundwater	4	16±2	≤4,320 ^g

^a Poliovirus retention is independent of flow rates between 4 and 20 L/minute for NanoCeram filters, but a constant flow rate, such as describe here, should be used for any single study (24). EPA may specify alternative flow rates for specific studies.

^b Consistent sample volumes should be used for any single study. EPA may specify alternative sample volumes for specific studies.

^c Turbidity and other factors may affect the volume collected during any sampling event. The sample duration must be increased to meet the specified or recommended volume during these situations. As an alternative, two cartridge filter modules may be used to obtain the specified volume.

^d This is a recommended value for final sewage effluents. There is no recommended volume for raw sewage.

^e The minimum specified volume is 300 L for surface waters.

^f The minimum specified volume is 1,500 L for treated tap or untreated groundwater.

^g For convenience, samples may be collected by starting the sampling at the end of a work day and stopping it in the morning of the next day.

METHOD 1615. Enterovirus and Norovirus occurrence in water

Table 3. MPN Program Default Settings for MPN Calculator

Item	Default Setting
Data Entry Mode	Keyboard
Dilution type	Standard 5-Fold Serial
Approximation type	Cornish & Fisher Limits
Confidence Level	95%
Number of Dilutions	1 (or 3 or actual number of dilutions)
Number of Tubes per dilution	10
Inoculum volume (mL)	The Inoculum Volume used (see section 11.2.4.5)

Table 4. Primers and TaqMan[®] Probes for Virus Detection by RT-qPCR

Virus group	Primer pairs ^{a,b}	TaqMan Probe ^{c,d}	Reference
<i>Enterovirus</i>	CCTCCGGCCCCCTGAATG ACCGGATGGCCAATCCAA	CGGAACCGACTACTTTGGGTGTCCGT	De Leon et al. (17) Monpocho et al.(30)
<i>Norovirus</i> GIA	GCCATGTTCCGITGGATG TCCTTAGACGCCATCATCAT	TGTGGACAGGAGATCGCAATCTC	Jothikumar et al. (23)
<i>Norovirus</i> GIB	CGTGGATGCGNTTCCAT CCTTAGACGCCATCATCATTTAC	TGGACAGGAGAYCGCRATCT	Butot et al. (12)
<i>Norovirus</i> GII	ATGTTTCAGRTGGATGAGRTTCTCWGA TCGACGCCATCTTCATTACACA	AGCACGTGGGAGGGGATCG	Butot et al. (12)
Hepatitis G	CGGCCAAAAGGTGGTGGATG CGACGAGCCTGACGTCGGG	AGGTCCCTCTGGCGCTTGTGGCGAG	Schlueter et al. (35)

^a EPA may specify additional or alternative primer sets for specific applications. Degenerate bases in primers and probes are as follows: N equals a mixture of all four nucleotides; R equals A + G; Y equals T + C; and W equals A + T.

^b The top primer in each set is the forward primer and the bottom primer is the reverse primer. All primer and probe sequences are 5' to 3'.

^c Probes are labeled on the 5'-end with 6-FAM and with TAMRA on the 3'-end.

^d Primers and probes are designated in Tables 6-10 by the first three letters of the virus name followed by F, R, or P for forward, reverse, and probe. GIA, GIB, or GII are also added to the norovirus designations.

METHOD 1615. Enterovirus and Norovirus occurrence in water

Table 5. RT Master Mix 1 and 2

Ingredient	Volume per Reaction (μL)^a	Final Concentration	Volume per Master Mix (μL)^b
RT Mix 1			
Random Primer	0.8	10 ng/μL (c. 5.6 μM)	77.6
Hepatitis G ^c	1		97
PCR grade water	14.2		1377.4
Total	16		1552
RT Mix 2			
10X PCR Buffer II	4	10 mM tris, pH 8.3, 50 mM KCL	388
25 mM MgCl ₂	4.8	3 mM	465.6
10 mM dNTPs	3.2	0.8 mM	310.4
100 mM DTT	4	10 mM	388
RNase Inhibitor ^d	1	0.5 units/μL	97
SuperScript II RT	0.3	1.6 units/μL	29.1
Total	17.3		1678.1

^a The volumes given are for 40 μL RT assays.

^b The volumes shown should be multiplied by the number of reactions to be performed plus an additional 0.5-1 reaction to account for losses during transfer of the master mix to tubes or plates. The amounts shown are sufficient for a 96 well PCR plate.

^c Hepatitis G Armored RNA is supplied as an untitered stock. The amount to use should be determined for each lot as described in section 13.5.8.2.

^d The amount shown is for a stock concentration of 20 unit/μL. The manufacturer's stock concentration varies between 20 and 40 units/μL. Amounts need to be adjusted for stocks with higher concentrations.

METHOD 1615. Enterovirus and Norovirus occurrence in water**Table 6. PCR Master Mix for Enterovirus Assay**

Ingredient	Volume per Reaction (μL)^a	Final Concentration	Volume per Master Mix (μL)^b
2X LightCycler 480 Probes Master Mix ^c	10	Proprietary	970
ROX reference dye ^d	0.4	0.5 mM	38.8
PCR grade water	1		97
EntF	0.6	300 nM	58.2
EntR	1.8	900 nM	174.6
EntP	0.2	100 nM	19.4
Total	14		1358

^a The volumes given are for using 6 μL of cDNA from Step 13.4.7 in a qPCR assay using a total qPCR volume of 20 μL.

^b The volumes shown should be multiplied by the number of reactions to be performed plus an additional 0.5-1 reaction to account for losses during transfer of the master mix to tubes or plates. The amounts shown are sufficient for a 96 well PCR plate.

^c 10X PCR Buffer II (2 μL/reaction), 25 mM MgCl₂ (5 μL/reaction), and AmpliTaq Gold (0.2 μL/reaction) can be substituted for the LightCycler 480 Probe Master mix.

^d This reagent is necessary for use with Applied Biosystems and similar instruments. It should be substituted with PCR grade water for use with the LightCycler and similar instruments.

Table 7. PCR Master Mix for Norovirus GIA Assay

Ingredient	Volume per Reaction (μL)^a	Final Concentration	Volume per Master Mix (μL)
2X LightCycler 480 Probes Master Mix	10	Proprietary	970
ROX reference dye	0.4	0.5 mM	38.8
PCR grade water	1.4		135.8
NorGIAF	1	500 nM	97
NorGIAR	1	500 nM	97
NorGIAP	0.2	100 nM	19.4
Total	14		1358

^a See table 6 for legends.

METHOD 1615. Enterovirus and Norovirus occurrence in water

Table 8. PCR Master Mix for Norovirus GIB Assay

Ingredient	Volume per Reaction (μL)^a	Final Concentration	Volume per Master Mix (μL)
2X LightCycler 480 Probes Master Mix	10	Proprietary	970
ROX reference dye	0.4	0.5 mM	38.8
PCR grade water	0.3		29.1
NorGIBF	1	500 nM	97
NorGIBR	1.8	900 nM	174.6
NorGIBP	0.5	250 nM	48.5
Total	14		1358

^a See table 6 for legends.

Table 9. PCR Master Mix for Norovirus GII Assay

Ingredient	Volume per Reaction (μL)^a	Final Concentration	Volume per Master Mix (μL)
2X LightCycler 480 Probes Master Mix	10	Proprietary	970
ROX reference dye	0.4	0.5 mM	38.8
PCR grade water	0.3		29.1
NorGIF	1	500 nM	97
NorGIR	1.8	900 nM	174.6
NorGIP	0.5	250 nM	48.5
Total	14		1358

^a See table 6 for legends.

METHOD 1615. Enterovirus and Norovirus occurrence in water

Table 10. PCR Master Mix for Hepatitis G Assay

Ingredient	Volume per Reaction (µL)^a	Final Concentration	Volume per Master Mix (µL)
2X LightCycler 480 Probes Master Mix	10	Proprietary	970
ROX reference dye	0.4	0.5 mM	38.8
PCR grade water	1.4		135.8
HepF	1	500 nM	97
HepR	1	500 nM	97
HepP	0.2	100 nM	19.4
Total	14		1358

^a See table 6 for legends.

Table 11. Mean Recovery and Coefficient of Variation Range

Variation Type	Mean Recovery Range	CV Range
Individual Analysts	36-98	34-157
Intralaboratory	36-85	58-131

METHOD 1615. Enterovirus and Norovirus occurrence in water

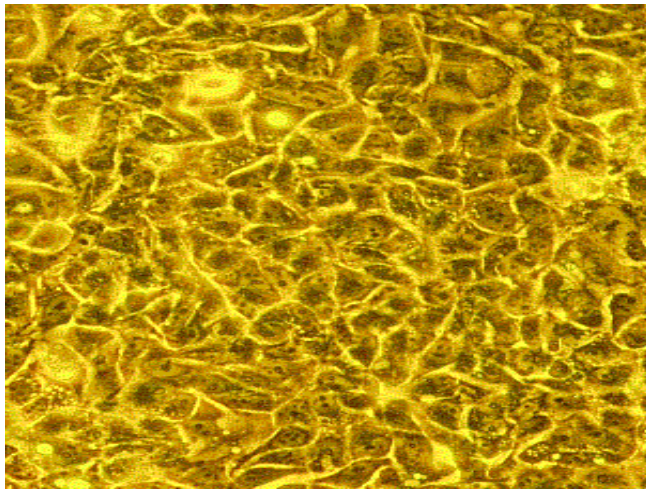


Figure 1. Uninfected BGM Cells

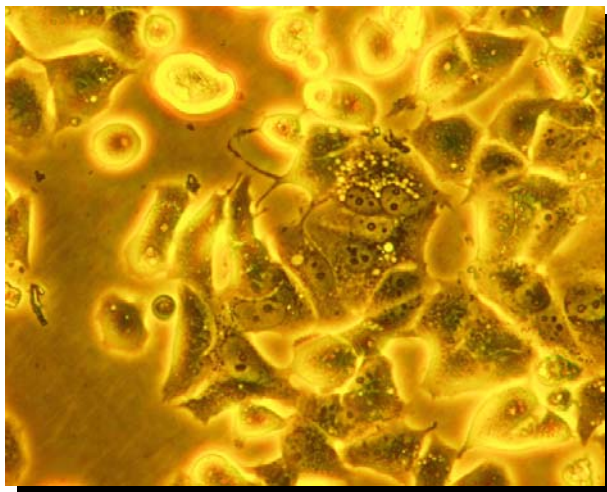


Figure 2. Early CPE from Poliovirus

METHOD 1615. Enterovirus and Norovirus occurrence in water

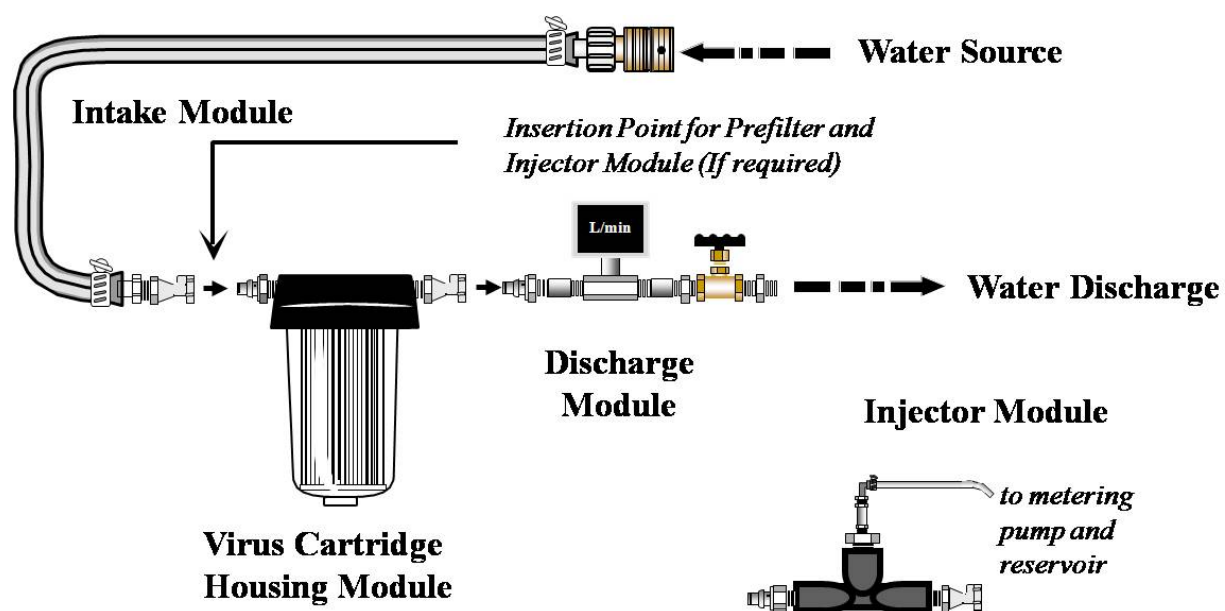


Figure 3. Sample Filtration Apparatus



Figure 4. Elution of an Electropositive Filter with Beef Extract

METHOD 1615. Enterovirus and Norovirus occurrence in water

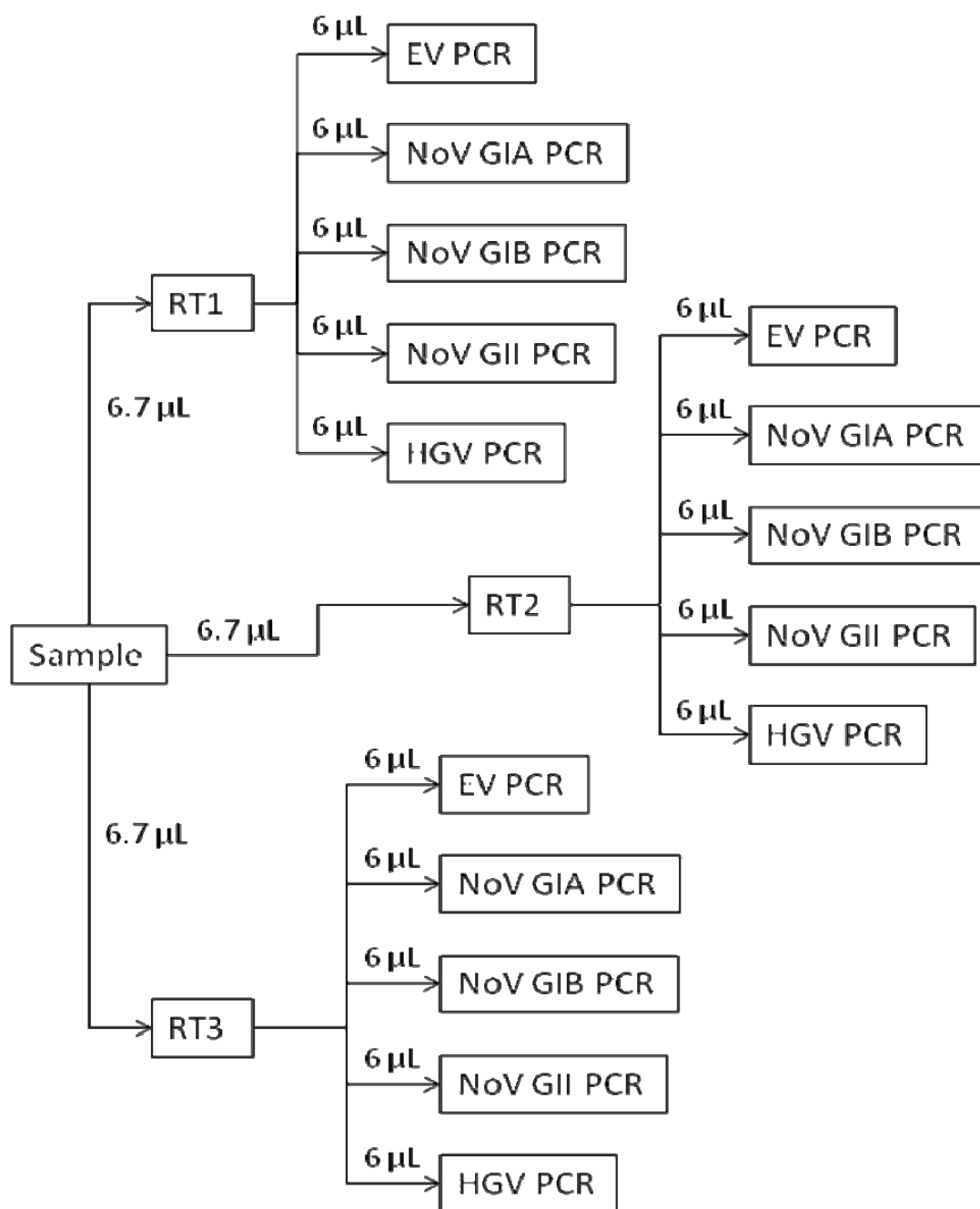


Figure 5. RT-qPCR Schematic

Each sample is reverse transcribed in triplicate (RT1, RT2, RT3) using 6.7 μL of extracted sample RNA for each RT assay in a 40 μL assay volume. Five qPCR assays (EV PCR, NoV GIA PCR, NoV GIB PCR, NoV GII PCR, and HGV PCR) are run from each of the triplicate RT reactions using 6 μL of cDNA for each qPCR assay.

METHOD 1615. Enterovirus and Norovirus occurrence in water

17. DATA SHEETS

17.1. SAMPLE DATA SHEET

Sample Data Sheet				
Sample Number/ID				
Utility Name				
Site Address				
City, State				
Sampler's Name ^a				
Water Type	<input type="checkbox"/> Surface waters	<input type="checkbox"/> Treated Surface or Groundwaters	<input type="checkbox"/> Untreated Groundwater	<input type="checkbox"/> Other (specify in comments section)
Location	<input type="checkbox"/> Treatment Plant/Pumping Station	<input type="checkbox"/> Distribution system	<input type="checkbox"/> Other (specify in comments section)	
	Start of Sampling Event		End of Sampling Event	
Date				
Time				
Totalizer Reading (L)				
Flow Rate (L/minute)				
Total Sample Volume (L)				
Water Parameter Readings				
Water Temperature				
pH				
Turbidity (NTU)				
Free Chlorine (mg/L)				
Quality Controls				
Flow meter model and serial number:				
Totalizer model and serial number:				
Date of last flow meter/totalizer calibration:				
Metering pump model and serial number				
Temperature meter model and serial number:				
pH meter model and serial number				
Turbidity meter model and serial number				
Chlorine test meter model and serial number				
Metering pump flow rate QC check performed	<input type="checkbox"/> Yes			
Comments:				

^a If any other individuals assist the sampler, include their name in the comments section and add the initials of the person who performed measurements after the recorded value.

METHOD 1615. Enterovirus and Norovirus occurrence in water

17.2. VIRUS DATA SHEET

VIRUS DATA SHEET			
Sample Number/ID:		Sample Date:	
Sample Arrival Date:		Hold Time Met (Y/N)	
Analytical Laboratory Name and ID:			
Analytical Laboratory Address:			
City:		State:	ZIP:
Analyst Name (Please print or type):			
Sample Batch Number:			
Date Eluted:		Time:	
Eluate Volume Recovered: L			
Date Concentrated:		Time:	
Centrifugation Speed (step 11.2.3.6): x g			
Final Concentrated Sample Volume (FCSV): mL			
Volume Of Original Water Sample Assayed (D) ^b : L			
Assay Sample Volume (S): mL			
Inoculum Volume: mL			
Final Inoculation Volume (If Used): mL			
<div> <div>Dates Assayed By CPE:</div> <div> <div>1st Passage</div> <div>2nd Passage</div> <div>3rd Passage (If necessary)</div> </div> </div>			
Subsample 1 ^a :			
MPN/L ^c :		95% Confidence Limits/L	
		Lower:	Upper:
Comments: Did a heavy floc form during the organic flocculation step? Yes___ No___ Was the floc difficult to dissolve? Yes___ No___ Other comments:			
Analyst Signature:			

^a Use a separate Virus Data Sheet if it is necessary to assay an additional subsample
^b e.g., 100 L of surface water or 500 L of finished or ground waters
^c Value calculated from the Quantitation of Total Culturable Virus form as described in the Virus Quantitation section.

METHOD 1615. Enterovirus and Norovirus occurrence in water

17.3. TOTAL CULTURABLE VIRUS DATA SHEET

TOTAL CULTURABLE VIRUS DATA SHEET					
Sample Number:					
Incubator Model and Serial Number:					
Passage	Sample Type	Confirmed ^a	Total Number of Replicates		
			Inoculated	Without CPE	With CPE
1st	Neg. Cont.				
	Pos. Cont.				
	Undiluted				
	1:5 Dil.				
	1:25 Dil.				
	1:125 Dil.				
2nd ^b	Neg. Cont.				
	Pos. Cont.				
	Undiluted				
	1:5 Dil.				
	1:25 Dil.				
	1:125 Dil.				
3rd ^c	Neg. Cont.				
	Pos. Cont.				
	Undiluted				
	1:5 Dil.				
	1:25 Dil.				
	1:125 Dil.				

^a Place a check (✓) next to the negative controls and dilutions that were confirmed.

^b A portion of medium from each 1st passage vessel, including negative controls, must be passaged again for confirmation. The terms "Undiluted," "1:5 Dilution" and "1:25 Dilution" under the 2nd and 3rd Passage headings refer to the original sample dilutions for the 1st passage.

^c Samples that were negative on the first passage and positive on the 2nd passage must be passaged a third time for confirmation. If a third passage is required, negative controls must be passaged again.

METHOD 1615. Enterovirus and Norovirus occurrence in water

17.4. QUANTITATION OF TOTAL CULTURABLE VIRUS DATA SHEET

QUANTITATION OF TOTAL CULTURABLE VIRUS DATA SHEET					
SAMPLE NUMBER:					
Sample	Number Replicates Inoculated	Number with CPE ^a	MPN/mL ^b	95% Confidence Limits/mL	
				Lower	Upper
Undiluted					
1:5 Dilution					
1:25 Dilution					
1:125 Dilution					
^a The number of flasks with confirmed CPE from the second passage (or third passage, if necessary). ^b The MPN/mL and 95% Confidence Limit values must be obtained using the computer program supplied by the U.S. EPA.					

METHOD 1615. Enterovirus and Norovirus occurrence in water

17.5. MOLECULAR VIRUS PROTOCOL DATA SHEET

MOLECULAR VIRUS PROTOCOL DATA SHEET				
Sample Number:				
Analytical Laboratory Name/ID:				
Analytical Laboratory Address:				
City:		State:		ZIP:
Analyst Name (Please print or type):				
Subsample Number:				
Sample Batch Number:				
Tertiary Concentration	Date:	Time:	Initials: ^a	
Concentrator Cat. No./Lot No.:				
Assay Sample Volume Concentrated:				^b mL
Final Tertiary Concentrated Volume:				μL
RNA Extraction	Date:	Time:	Initials:	
RNA Extraction Kit Cat. No./Lot No.:				
Amount Used For RNA Extraction:				μL
RNA Extract Final Volume:				μL
Reverse Transcription (RT) Step				
Master Mixes Prepared	Date:	Time:	Initials:	
RNA Extract Volume Used For RT:				μL
RT Samples Run:	Date:	Time:	Initials:	
Thermal Cycler Used: ^c				
PCR Step				
Master Mixes Prepared:	Date:	Time:	Initials:	
Volume Of RT Used For PCR:				μL
PCR Samples Run	Date:	Time:	Initials:	
Thermal Cycler/Real Time Instrument Used: ^c				
Standard Curves				
Enterovirus	Lot # ^d	Eff.: ^e	R ²	SD ^f
Norovirus GI	Lot #	Eff.	R ²	SD
Norovirus GII	Lot #	Eff.	R ²	SD
^a Record the initials of the analyst at the time this procedure is performed. ^b The Assay Sample Volume should be the same as recorded on the Virus Data Sheet. ^c Record the make and model of thermal cycler. ^d Assign a new lot number to each standard curve run. ^e Percent efficiency ^f Record the largest standard deviation among the different concentrations of the standard curve lot.				

METHOD 1615. Enterovirus and Norovirus occurrence in water

17.6. MOLECULAR VIRUS RESULTS DATA SHEET

MOLECULAR VIRUS RESULTS DATA SHEET				
Sample Number:				
Analytical Laboratory Name/ID:				
Analytical Laboratory Address:				
City:		State:		ZIP:
Analyst Name (Please print or type):				
RT-qPCR Results:				
All No Template Controls Negative:		Yes		No ^a
Sample Type	Target Value (Sd) ^b		Actual Value ^{a,c}	
Inhibitor Control				
Enterovirus Calibrator				
<i>Norovirus</i> GI Calibrator				
<i>Norovirus</i> GII Calibrator				
Run Number: ^d				
If Required, Dilution Used In Calibration Of Sample Concentration:				
Replicate ^e	1	2	3	Mean (SD) ^b
Genomic Copy Values				
Genomic Copies per L: ^f				
<p>^a If any no template controls are positive or the inhibition control or calibrator falls outside specification limits, the samples must be re-run; however, each run should be recorded on this data sheet.</p> <p>^b Record the mean and the standard deviation values for the sample type. For water samples, include non-detects (zeros) in the calculation of the mean.</p> <p>^c Record the actual Cq value obtained for the sample type</p> <p>^d A serial record identification of samples that have to be re-run.</p> <p>^e If more than three replicates are used, record the data from the additional replicates onto another Molecular Virus Results Data Sheet.</p> <p>^f Calculate the Genomic Copies per L using Equation 5. For water samples with a mean value of zero, report the Genomic Copies per L as less than or equal to the detection limit.</p>				

METHOD 1615. Enterovirus and Norovirus occurrence in water

18. REFERENCES

1. 1999. Biosafety in Microbiological and Biomedical Laboratories, Fourth ed. U.S. Department of Health and Human Services, Washington, D.C.
2. 1998. Standard Methods for the Examination of Water and Wastewater, 20th ed. American Public Health Association, Washington, D.C.
3. **Abzug, M. J.** 2008. The enteroviruses: an emerging infectious disease? The real, the speculative and the really speculative. Springer, New York.
4. **Amvrosieva, T. V., N. V. Paklonskaya, A. A. Biazruchka, O. N. Kazinetz, Z. F. Bohush, and E. G. Fisenko.** 2006. Enteroviral infection outbreak in the Republic of Belarus: principal characteristics and phylogenetic analysis of etiological agents. *Cent Eur J Public Health* **14**:67-73.
5. **Astrom, J., S. Pettersson, O. Bergstedt, T. J. Pettersson, and T. A. Stenstrom.** 2007. Evaluation of the microbial risk reduction due to selective closure of the raw water intake before drinking water treatment. *J Water Health* **5 Suppl 1**:81-97.
6. **Berg, G., R. S. Safferman, D. R. Dahling, D. Berman, and C. J. Hurst.** 1984. USEPA Manual of Methods for Virology. U.S. Environmental Protection Agency, Cincinnati, OH.
7. **Borchardt, M. A., K. R. Bradbury, E. C. Alexander, Jr., R. J. Kolberg, S. C. Alexander, J. R. Archer, L. A. Braatz, B. M. Forest, J. A. Green, and S. K. Spencer.** 2010. Norovirus Outbreak Caused by a New Septic System in a Dolomite Aquifer. Ground Water.
8. **Borchardt, M. A., K. R. Bradbury, M. B. Gotkowitz, J. A. Cherry, and B. L. Parker.** 2007. Human enteric viruses in groundwater from a confined bedrock aquifer. *Environ Sci Technol* **41**:6606-12.
9. **Borchardt, M. A., S. K. Spencer, B. A. Kieke Jr., E. Lambertini, and F. J. Loge.** Human viruses in groundwater: association with community rates of acute gastrointestinal illness. Submitted.
10. **Bustin, S. A.** 2010. Developments in real-time PCR research and molecular diagnostics. *Expert Rev Mol Diagn* **10**:713-5.
11. **Bustin, S. A., V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele, and C. T. Wittwer.** 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* **55**:611-22.
12. **Butot, S., F. S. Le Guyader, J. Krol, T. Putallaz, R. Amoroso, and G. Sanchez.** 2010. Evaluation of various real-time RT-PCR assays for the detection and quantitation of human norovirus. *J Virol Methods* **167**:90-4.
13. **Chappell, J. D., R. Duncan, P. P. C. Mertens, and T. S. Dermody.** 2005. Genus Orthoreovirus, p. 455–465. *In* C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger, and L. A. Ball (ed.), *Virus Taxonomy Eighth Report of the International Committee on Taxonomy of Viruses*. Elsevier Academic Press, San Diego.
14. **Chapron, C. D., N. A. Ballester, J. H. Fontaine, C. N. Frades, and A. B. Margolin.** 2000. Detection of astroviruses, enteroviruses, and adenovirus types 40 and 41 in surface waters collected and evaluated by the information collection rule and an integrated cell culture-nested PCR procedure. *Appl Environ Microbiol* **66**:2520-5.

METHOD 1615. Enterovirus and Norovirus occurrence in water

15. **Chua, K. B., K. Voon, G. Cramer, H. S. Tan, J. Rosli, J. A. McEachern, S. Suluraju, M. Yu, and L. F. Wang.** 2008. Identification and characterization of a new orthoreovirus from patients with acute respiratory infections. *PLoS ONE* **3**:e3803.
16. **Dahling, D. R., and B. A. Wright.** 1986. Optimization of the BGM cell line culture and viral assay procedures for monitoring viruses in the environment. *Appl Environ Microbiol* **51**:790-812.
17. **De Leon, R., Y. S. C. Shieh, R. Baric, and M. D. Sobsey.** 1990. Detection of enteroviruses and hepatitis A virus in environmental samples by gene probes and polymerase chain reaction. *Am Water Works Assoc Proc* **1989**:833-853.
18. **Fout, G. S., B. C. Martinson, M. W. Moyer, and D. R. Dahling.** 2003. A multiplex reverse transcription-PCR method for detection of human enteric viruses in groundwater. *Appl Environ Microbiol* **69**:3158-64.
19. **Fout, G. S., F. W. Schaefer, 3rd, J. W. Messer, D. R. Dahling, and R. E. Stetler.** 1996. ICR microbial laboratory manual. U.S. Environmental Protection Agency, Washington, D.C.
20. **Harris, J. P., W. J. Edmunds, R. Pebody, D. W. Brown, and B. A. Lopman.** 2008. Deaths from norovirus among the elderly, England and Wales. *Emerg Infect Dis* **14**:1546-52.
21. **Heid, C. A., J. Stevens, K. J. Livak, and P. M. Williams.** 1996. Real time quantitative PCR. *Genome Res* **6**:986-94.
22. **Hewitt, J., D. Bell, G. C. Simmons, M. Rivera-Aban, S. Wolf, and G. E. Greening.** 2007. Gastroenteritis outbreak caused by waterborne norovirus at a New Zealand ski resort. *Appl Environ Microbiol* **73**:7853-7.
23. **Jothikumar, N., J. A. Lowther, K. Henshilwood, D. N. Lees, V. R. Hill, and J. Vinje.** 2005. Rapid and sensitive detection of noroviruses by using TaqMan-based one-step reverse transcription-PCR assays and application to naturally contaminated shellfish samples. *Appl Environ Microbiol* **71**:1870-5.
24. **Karim, M. R., E. R. Rhodes, N. E. Brinkman, L. Wymer, and G. S. Fout.** 2009. New electropositive filter for concentrating enteroviruses and noroviruses from large volumes of water. *Appl Environ Microbiol* **75**:2393-2399.
25. **Khetsuriani, N., A. Lamonte-Fowlkes, S. Oberst, and M. A. Pallansch.** 2006. Enterovirus surveillance--United States, 1970-2005. *MMWR Surveill Summ* **55**:1-20.
26. **Kim, S. H., D. S. Cheon, J. H. Kim, D. H. Lee, W. H. Jheong, Y. J. Heo, H. M. Chung, Y. Jee, and J. S. Lee.** 2005. Outbreaks of gastroenteritis that occurred during school excursions in Korea were associated with several waterborne strains of norovirus. *J Clin Microbiol* **43**:4836-9.
27. **Lambertini, E., S. K. Spencer, P. D. Bertz, F. J. Loge, B. A. Kieke, and M. A. Borchardt.** 2008. Concentration of enteroviruses, adenoviruses, and noroviruses from drinking water by use of glass wool filters. *Appl Environ Microbiol* **74**:2990-6.
28. **Lee, G., C. Lee, C. Park, and S. Jeong.** 2008. Detection and molecular characterization of enteroviruses in Korean surface water by using integrated cell culture multiplex RT-PCR. *Biomed Environ Sci* **21**:425-31.
29. **Malherbe, H. H., and M. Strickland-Cholmley.** 1980. *Viral Cytopathology*. CRC Press, Boca Raton, FL.
30. **Monpoeho, S., A. Dehee, B. Mignotte, L. Schwartzbrod, V. Marechal, J. C. Nicolas, S. Billaudel, and V. Ferre.** 2000. Quantification of enterovirus RNA in sludge samples using single tube real-time RT-PCR. *Biotechniques* **29**:88-93.

METHOD 1615. Enterovirus and Norovirus occurrence in water

31. **Parshionikar, S., I. Laseke, and G. S. Fout.** 2010. Use of propidium monoazide in reverse transcriptase PCR to distinguish between infectious and noninfectious enteric viruses in water samples. *Appl Environ Microbiol* **76**:4318-26.
32. **Parshionikar, S. U., J. Cashdollar, and G. S. Fout.** 2004. Development of homologous viral internal controls for use in RT-PCR assays of waterborne enteric viruses. *J Virol Methods* **121**:39-48.
33. **Puig, M., J. Jofre, F. Lucena, A. Allard, G. Wadell, and R. Girones.** 1994. Detection of adenoviruses and enteroviruses in polluted waters by nested PCR amplification. *Appl Environ Microbiol* **60**:2963-70.
34. **Reynolds, K. A., C. P. Gerba, and I. L. Pepper.** 1996. Detection of infectious enteroviruses by an integrated cell culture-PCR procedure. *Appl Environ Microbiol* **62**:1424-7.
35. **Schlueter, V., S. Schmolke, K. Stark, G. Hess, B. Ofenloch-Haehnle, and A. M. Engel.** 1996. Reverse transcription-PCR detection of hepatitis G virus. *J Clin Microbiol* **34**:2660-4.
36. **Sen, K., G. S. Fout, R. Haugland, C. Moulton, A. Grimm, G. Di Giovanni, M. A. Feige, J. B. Best, G. Lott, J. Scheller, E. Reilly, K. Connell, and M. Marshall.** 2004. Quality assurance/quality control guidance for laboratories performing PCR analyses on environmental samples. U.S. Environmental Protection Agency, Cincinnati, OH.
37. **Straub, T. M., R. A. Bartholomew, C. O. Valdez, N. B. Valentine, A. Dohnalkova, R. M. Ozanich, C. J. Bruckner-Lea, and D. R. Call.** Human norovirus infection of Caco-2 cells grown as a 3-dimensional tissue structure. *J Water Health* **Accepted for publication September 9, 2010**.
38. **Straub, T. M., K. Honer zu Bentrup, P. Orosz-Coghlan, A. Dohnalkova, B. K. Mayer, R. A. Bartholomew, C. O. Valdez, C. J. Bruckner-Lea, C. P. Gerba, M. Abbaszadegan, and C. A. Nickerson.** 2007. In vitro cell culture infectivity assay for human noroviruses. *Emerg Infect Dis* **13**:396-403.
39. **Tsai, Y. L., M. D. Sobsey, L. R. Sangermano, and C. J. Palmer.** 1993. Simple method of concentrating enteroviruses and hepatitis A virus from sewage and ocean water for rapid detection by reverse transcriptase-polymerase chain reaction. *Appl Environ Microbiol* **59**:3488-91.
40. **Wade, T. J., E. Sams, K. P. Brenner, R. Haugland, E. Chern, M. Beach, L. Wymer, C. C. Rankin, D. Love, Q. Li, R. Noble, and A. P. Dufour.** 2010. Rapidly measured indicators of recreational water quality and swimming-associated illness at marine beaches: a prospective cohort study. *Environ Health* **9**:66.
41. **Yoder, J., V. Roberts, G. F. Craun, V. Hill, L. Hicks, N. T. Alexander, V. Radke, R. L. Calderon, M. C. Hlavsa, M. J. Beach, and S. L. Roy.** 2008. Surveillance for waterborne disease and outbreaks associated with drinking water and water not intended for drinking -United States, 2005-2006. *Morb Mortal Wkly Rep* **57**:39-69.
42. **Zheng, D. P., T. Ando, R. L. Fankhauser, R. S. Beard, R. I. Glass, and S. S. Monroe.** 2006. Norovirus classification and proposed strain nomenclature. *Virology* **346**:312-23.

SCIENCE



PRESORTED STANDARD
POSTAGE & FEES PAID
EPA
PERMIT NO. G-35

Office of Research and Development
Cincinnati, OH 45268

Official Business
Penalty for Private Use
\$300